PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 24 May 2000 (24.05.00)	
International application No. PCT/US99/24922	**Applicant's or agent's file reference** BB1208 PCT
International filing date (day/month/year) 22 October 1999 (22.10.99)	**Priority date** (day/month/year) 23 October 1998 (23.10.98)
Applicant MCCUTCHEN, Billy, F. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

28 April 2000 (28.04.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer F. Baechler
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

NOV 30 2000

RECEIVED

NOV 29 2000

PATENT RECORDS CENTER

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
E.I. DU PONT DE NEMOURS AND COMPANY
Legal Patent Records Center
Attn. FEULNER, GREGORY J.
1007 Market Street
Wilmington, Delaware 19898
UNITED STATES OF AMERICA

Date of mailing *(day/month/year)* 23/11/2000	
Applicant's or agent's file reference BB1208 PCT	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US 99/24922	International filing date *(day/month/year)* 22/10/1999
Applicant E. I. DU PONT DE NEMOURS AND COMPANY et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Véronique Baillou

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

NOTES TO FORM PCT/ISA/220 (continued)

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments and any accompanying statement, under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the time of filing the amendments (and any statement) with the International Bureau, also file with the International Preliminary Examining Authority a copy of such amendments (and of any statement) and, where required, a translation of such amendments for the procedure before that Authority (see Rules 55.3(a) and 62.2, first sentence). For further information, see the Notes to the demand form (PCT/IPEA/401).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
BB1208 PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 99/ 24922	22/10/1999	23/10/1998
Applicant		
E. I. DU PONT DE NEMOURS AND COMPANY et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 6 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☒ contained in the international application in written form.

☒ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readble form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☒ **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

☒ as suggested by the applicant.

☐ None of the figures.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/24922

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1 - 20 (partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion alpha toxin XIV polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:2 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

2. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion alpha toxin XIV polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:4 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

3. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion alpha toxin XIV polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:6 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

4. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion neurotoxin I polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:9 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

5. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion depressant toxin LqhIT2 polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:12 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

6. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion depressant toxin LqhIT2 polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:14 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

7. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion depressant toxin LqhIT2 polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:16 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/24922

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C12N15/86 C12N7/01 C12N5/10 C07K14/435
A01N63/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, STRAND, MEDLINE, EMBASE, BIOSIS, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 36712 A (E.I. DU PONT DE NEMOURS AND COMPANY) 21 November 1996 (1996-11-21) page 3, line 10 - line 35; examples 1-4 ---	1-20
A	WO 96 36221 A (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 21 November 1996 (1996-11-21) page 6, line 28 -page 19, line 2 ---	1-20
A	SAUTIÈRE, P. ET AL.: "New toxins acting on sodium channels from the scorpion Leiurus Quinquestriatus Hebraeus suggest a clue to mammalian vs insect selectivity" TOXICON, vol. 36, no. 8, August 1998 (1998-08), pages 1141-1154, XP000916899 page 1145 -page 1152 'Results and Discussion' --- -/--	1-20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 July 2000	23. 11. 00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Donath, C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/24922

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MOSKOWITZ, H. ET AL.: "A depressant insect-selective toxin analog from the venom of the scorpion Leiurus quinquestriatus hebraeus, purification and structure/function characterization" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 254, no. 1, 15 May 1998 (1998-05-15), pages 44-49, XP000919258 page 45 -page 49 'Results' and 'Discussion' ---	1-20
A	BOUHAOUALA-ZAHAR, B. ET AL.: "A recombinant insect-specific alpha-toxin of Buthus occitanus tunetanus scorpion confers protection against homologous mammal toxins" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 238, no. 3, 15 June 1996 (1996-06-15), pages 653-660, XP000919257 page 655 -page 659 'Results' and 'Discussion' -----	1-20

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/24922

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9636712 A	21-11-1996	AU 723612 B	31-08-2000
		AU 5865196 A	29-11-1996
		BR 9608741 A	06-07-1999
		CN 1184504 A	10-06-1998
		EP 0826047 A	04-03-1998
		JP 10509596 T	22-09-1998
		NZ 308772 A	29-04-1999
		US 6096304 A	01-08-2000
WO 9636221 A	21-11-1996	US 5756340 A	26-05-1998
		AU 710774 B	30-09-1999
		AU 5788796 A	29-11-1996
		BR 9608474 A	13-10-1999
		CN 1185718 A	24-06-1998
		EP 0838999 A	06-05-1998
		JP 11501521 T	09-02-1999
		NZ 308294 A	27-05-1998

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
BB1208 PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 99/ 24922	22/10/1999	23/10/1998
Applicant		
E. I. DU PONT DE NEMOURS AND COMPANY et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 6 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☒ contained in the international application in written form.

☒ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readble form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☒ **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

☒ as suggested by the applicant. ☐ None of the figures.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/24922

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1 - 20 (partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion alpha toxin XIV polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:2 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

2. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion alpha toxin XIV polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:4 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

3. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion alpha toxin XIV polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:6 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

4. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion neurotoxin I polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:9 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

5. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion depressant toxin LqhIT2 polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:12 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

6. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion depressant toxin LqhIT2 polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:14 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

7. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion depressant toxin LqhIT2 polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:16 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

INTERNATIONAL SEARCH REPORT

[redacted]ational Application No
PCT/US 99/24922

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C12N15/86 C12N7/01 C12N5/10 C07K14/435
A01N63/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, STRAND, MEDLINE, EMBASE, BIOSIS, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 36712 A (E.I. DU PONT DE NEMOURS AND COMPANY) 21 November 1996 (1996-11-21) page 3, line 10 - line 35; examples 1-4 ---	1-20
A	WO 96 36221 A (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 21 November 1996 (1996-11-21) page 6, line 28--page 19, line 2 ---	1-20
A	SAUTIÈRE, P. ET AL.: "New toxins acting on sodium channels from the scorpion Leiurus Quinquestriatus Hebraeus suggest a clue to mammalian vs insect selectivity" TOXICON, vol. 36, no. 8, August 1998 (1998-08), pages 1141-1154, XP000916899 page 1145 -page 1152 'Results and Discussion' --- -/--	1-20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 July 2000	2[illegible]

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Donath, C

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MOSKOWITZ, H. ET AL.: "A depressant insect-selective toxin analog from the venom of the scorpion Leiurus quinquestriatus hebraeus, purification and structure/function characterization" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 254, no. 1, 15 May 1998 (1998-05-15), pages 44-49, XP000919258 page 45 -page 49 'Results' and 'Discussion' ---	1-20
A	BOUHAOUALA-ZAHAR, B. ET AL.: "A recombinant insect-specific alpha-toxin of Buthus occitanus tunetanus scorpion confers protection against homologous mammal toxins" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 238, no. 3, 15 June 1996 (1996-06-15), pages 653-660, XP000919257 page 655 -page 659 'Results' and 'Discussion' -----	1-20

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/24922

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9636712 A	21-11-1996	AU 723612 B	31-08-2000
		AU 5865196 A	29-11-1996
		BR 9608741 A	06-07-1999
		CN 1184504 A	10-06-1998
		EP 0826047 A	04-03-1998
		JP 10509596 T	22-09-1998
		NZ 308772 A	29-04-1999
		US 6096304 A	01-08-2000
WO 9636221 A	21-11-1996	US 5756340 A	26-05-1998
		AU 710774 B	30-09-1999
		AU 5788796 A	29-11-1996
		BR 9608474 A	13-10-1999
		CN 1185718 A	24-06-1998
		EP 0838999 A	06-05-1998
		JP 11501521 T	09-02-1999
		NZ 308294 A	27-05-1998

PCT

WORLD I[redacted]RTY ORGANIZATION
[redacted] Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C07K 14/00

A2

(11) International Publication Number: WO 00/24772

(43) International Publication Date: 4 May 2000 (04.05.00)

(21) International Application Number: PCT/US99/24922

(22) International Filing Date: 22 October 1999 (22.10.99)

(30) Priority Data:
60/105,404 23 October 1998 (23.10.98) US

(71) Applicant (*for all designated States except US*): E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): MCCUTCHEN, Billy, F. [US/US]; 421 Christiana Mills Drive, Newark, DE 19808 (US). HERRMANN, Rafael [IL/US]; Apartment 405, 3120 Naamans Road, Wilmington, DE 19810 (US).

(74) Agent: FEULNER, Gregory, J.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).

(81) Designated States: AE, AL, AU, BA, BB, BG, BR, CA, CN, CR, CU, CZ, DM, EE, GD, GE, HR, HU, ID, IL, IN, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ZA, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: SCORPION TOXINS

```
                                        ↓
SEQ ID NO:7  MSSL-MISTAMKGKAPY-RQVRDGYIAQPHN [C] AYH [C] LKISSG [C] DTL [C] KENGATSGH [C] GHK
SEQ ID NO:2  --------LALLFMTGVES-VRDGYIAQPEN [C] VYH [C] IPD--- [C] DTL [C] KDNGGTGGH [C] GFK
SEQ ID NO:4  -------SLALLFMTGVES-VRDGYIAKPEN [C] AHH [C] FPGSSG [C] DTL [C] KENGGTGGH [C] GFK
SEQ ID NO:6  MNHLVMISLALLFMTGVESGVRDGYIAQPEN [C] VYH [C] FPGSPG [C] DTL [C] KENGASSGH [C] GFK
             1                                                                          60

SEQ ID NO:7  SGHGSA [C] W C KDLPDKVGIIVHGEK C HR
SEQ ID NO:2  LGHGIA [C] W C NALPDNVGIIVDGVK C HK
SEQ ID NO:4  VGHGTA [C] W C NALPDKVGIIVDGVK C HR
SEQ ID NO:6  EGHGLA [C] W C NDLPDKVGIIVEGEK C HK
             61                                87
```

(57) Abstract

This invention relates to an isolated nucleic acid fragment encoding a scorpion sodium channel agonist. The invention also relates to the construction of a chimeric gene encoding all or a portion of the scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

BB 1208

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C07K 14/00

A2

(11) International Publication Number: WO 00/24772

(43) International Publication Date: 4 May 2000 (04.05.00)

(21) International Application Number: PCT/US99/24922

(22) International Filing Date: 22 October 1999 (22.10.99)

(30) Priority Data:
60/105,404 23 October 1998 (23.10.98) US

(71) Applicant *(for all designated States except US)*: E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MCCUTCHEN, Billy, F. [US/US]; 421 Christiana Mills Drive, Newark, DE 19808 (US). HERRMANN, Rafael [IL/US]; Apartment 405, 3120 Naamans Road, Wilmington, DE 19810 (US).

(74) Agent: FEULNER, Gregory, J.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).

(81) Designated States: AE, AL, AU, BA, BB, BG, BR, CA, CN, CR, CU, CZ, DM, EE, GD, GE, HR, HU, ID, IL, IN, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ZA, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: SCORPION TOXINS

```
                                        ▼
SEQ ID NO:7  MSSL-MISTAMKGKAPY-RQVRDGYIAQPHN C AYH C LKISSG C DTL C KENGATSGH C GHK
SEQ ID NO:2  --------LALLFMTGVES-VRDGYIAQPEN C VYH C IPD--- C DTL C KDNGGTGGH C GFK
SEQ ID NO:4  -------SLALLFMTGVES-VRDGYIAKPEN C AHH C FPGSSG C DTL C KENGGTGGH C GFK
SEQ ID NO:6  MNHLVMISLALLFMTGVESGVRDGYIAQPEN C VYH C FPGSPG C DTL C KENGASSGH C GFK
             1                                                                  60

SEQ ID NO:7  SGHGSA C W C KDLPDKVGIIVHGEK C HR
SEQ ID NO:2  LGHGIA C W C NALPDNVGIIVDGVK C HK
SEQ ID NO:4  VGHGTA C W C NALPDKVGIIVDGVK C HR
SEQ ID NO:6  EGHGLA C W C NDLPDKVGIIVEGEK C HK
             61                            87
```

(57) Abstract

This invention relates to an isolated nucleic acid fragment encoding a scorpion sodium channel agonist. The invention also relates to the construction of a chimeric gene encoding all or a portion of the scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 May 2000 (04.05.2000)

PCT

(10) International Publication Number
WO 00/24772 A3

(51) International Patent Classification[7]: C12N 15/12, 15/86, 7/01, 5/10, C07K 14/435, A01N 63/02

(21) International Application Number: PCT/US99/24922

(22) International Filing Date: 22 October 1999 (22.10.1999)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/105,404 23 October 1998 (23.10.1998) US

(71) Applicant *(for all designated States except US)*: **E.I. DU PONT DE NEMOURS AND COMPANY** [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **MCCUTCHEN, Billy, F.** [US/US]; 421 Christiana Mills Drive, Newark, DE 19808 (US). **HERRMANN, Rafael** [IL/US]; Apartment 405, 3120 Naamans Road, Wilmington, DE 19810 (US).

(74) Agent: **FEULNER, Gregory, J.**; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).

(81) Designated States *(national)*: AE, AL, AU, BA, BB, BG, BR, CA, CN, CR, CU, CZ, DM, EE, GD, GE, HR, HU, ID, IL, IN, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ZA.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

(88) Date of publication of the international search report:
1 February 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: SCORPION TOXINS

```
                                                    ↓
SEQ ID NO:7  MSSL-MISTAMKGKAPY-RQVRDGYIAQPHN C AYH C LKISSG C DTL C KENGATSGH C GHK
SEQ ID NO:2  --------LALLFMTGVES-VRDGYIAQPEN C VYH C IPD--- C DTL C KDNGGTGGH C GFK
SEQ ID NO:4  --------SLALLFMTGVES-VRDGYIAKPEN C AHH C FPGSSG C DTL C KENGGTGGH C GFK
SEQ ID NO:6  MNHLVMISLALLFMTGVESGVRDGYIAQPEN C VYH C FPGSPG C DTL C KENGASSGH C GFK
                1                                                              60

SEQ ID NO:7  SGHGSA C W C KDLPDKVGIIVHGEK C HR
SEQ ID NO:2  LGHGIA C W C NALPDNVGIIVDGVK C HK
SEQ ID NO:4  VGHGTA C W C NALPDKVGIIVDGVK C HR
SEQ ID NO:6  EGHGLA C W C NDLPDKVGIIVEGEK C HK
               61                          87
```

(57) Abstract: This invention relates to an isolated nucleic acid fragment encoding a scorpion sodium channel agonist. The invention also relates to the construction of a chimeric gene encoding all or a portion of the scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

WO 00/24772 A3

INTERNATIONAL SEARCH REPORT

Inte[illegible]ional Application No
PCT/US 99/24922

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C12N15/86 C12N7/01 C12N5/10 C07K14/435
A01N63/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, STRAND, MEDLINE, EMBASE, BIOSIS, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 36712 A (E.I. DU PONT DE NEMOURS AND COMPANY) 21 November 1996 (1996-11-21) page 3, line 10 - line 35; examples 1-4 ---	1-20
A	WO 96 36221 A (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 21 November 1996 (1996-11-21) page 6, line 28 -page 19, line 2 ---	1-20
A	SAUTIÈRE, P. ET AL.: "New toxins acting on sodium channels from the scorpion Leiurus Quinquestriatus Hebraeus suggest a clue to mammalian vs insect selectivity" TOXICON, vol. 36, no. 8, August 1998 (1998-08), pages 1141-1154, XP000916899 page 1145 -page 1152 'Results and Discussion' --- -/--	1-20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 July 2000	23.11.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Donath, C

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MOSKOWITZ, H. ET AL.: "A depressant insect-selective toxin analog from the venom of the scorpion Leiurus quinquestriatus hebraeus, purification and structure/function characterization" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 254, no. 1, 15 May 1998 (1998-05-15), pages 44-49, XP000919258 page 45 -page 49 'Results' and 'Discussion' ---	1-20
A	BOUHAOUALA-ZAHAR, B. ET AL.: "A recombinant insect-specific alpha-toxin of Buthus occitanus tunetanus scorpion confers protection against homologous mammal toxins" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 238, no. 3, 15 June 1996 (1996-06-15), pages 653-660, XP000919257 page 655 -page 659 'Results' and 'Discussion' -----	1-20

2

INTERNATIONAL SEARCH REPORT

national application No.
PCT/US 99/24922

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1 - 20 (partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion alpha toxin XIV polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:2 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

2. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion alpha toxin XIV polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:4 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

3. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion alpha toxin XIV polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:6 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

4. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion neurotoxin I polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:9 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

5. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion depressant toxin LqhIT2 polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:12 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

6. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion depressant toxin LqhIT2 polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:14 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

7. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion depressant toxin LqhIT2 polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:16 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/24922

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9636712 A	21-11-1996	AU	723612 B	31-08-2000
		AU	5865196 A	29-11-1996
		BR	9608741 A	06-07-1999
		CN	1184504 A	10-06-1998
		EP	0826047 A	04-03-1998
		JP	10509596 T	22-09-1998
		NZ	308772 A	29-04-1999
		US	6096304 A	01-08-2000
WO 9636221 A	21-11-1996	US	5756340 A	26-05-1998
		AU	710774 B	30-09-1999
		AU	5788796 A	29-11-1996
		BR	9608474 A	13-10-1999
		CN	1185718 A	24-06-1998
		EP	0838999 A	06-05-1998
		JP	11501521 T	09-02-1999
		NZ	308294 A	27-05-1998

JC Rec'd PCT/PTO 09 APR 2001
PCT/US99/24922

TITLE
SCORPION TOXINS

FIELD OF THE INVENTION

This invention is in the field of molecular biology. More specifically, this invention pertains to nucleic acid fragments encoding scorpion toxins that are sodium channel agonists.

BACKGROUND OF THE INVENTION

Alpha neurotoxins are short, single-chain, polypeptides crosslinked by four disulfide bridges, and responsible for insect and mammal poisonings. These neurotoxins show variability in their apparent toxicity, in their primary structures, and in their binding features to neuronal membrane preparations (Dufton and Rochat (1984) *J. Mol. Evol. 20*:120-127). Despite differences in their primary structures and phylogenetic selectivity, scorpion neurotoxins affecting sodium (Na) channels are closely related in their spatial arrangement. And in their compact globular structure kept rigid by the four disulfide bridges (Miranda et al. (1970) *Eur. J. Biochem, 16*:514-523; and Fontecilla-Camps (1989) *J. Mol. Evol. 29*:63-67).

Zilbergberg and coworkers determined that single amino acid residues are important for receptor binding and for biological activity of scorpion Na channel toxins (Zilbergberg et al. (1997) *J. Biol. Chem. 272*:14810-14816). As examples, the lysine at position 8 of LqhIT was demonstrated to be necessary for binding activity and toxicity without change in overall structure. A substantial decrease in biological activity without a significant change in structure was found when the aromatic amino acid phenylalanine, at position 17, was substituted for glycine. Conversely, changes in structure are not necessarily associated with differences in toxicity as demonstrated when tyrosine at position 49 was changed to leucine.

While potassium (K) channels have been shown to be central to heart function, the role of chlorine- (Cl) and Na-channels in this activity is less clear (Johnson et al.. (1998) *J. Neurogent. 12*:1-24). Sodium entry hyperpolarizes the cell, producing indirect, Na-dependent changes of calcium transport (Friedman (1998) *Annu. Rev. Physiol. 60*:179-197). Abnormal influx of calcium is thought to be very important in the pathogenesis of several central nervous system disorders in vertebrates, including stroke damage, epilepsy, and the neuronal death associated with chronic epilepsy.

Excitatory amino acids, most notably glutamate and aspartate, are the predominant excitatory neurotransmitter in the vertebrate (including human) central nervous system. These amino acids are released from presynaptic nerve terminals and, after diffusing across the synaptic cleft, contact special receptor molecules in the postsynaptic cell membrane. These receptors indirectly influence the flow of various ions across the cell membrane and

thus contribute to production of an electrical response to the chemical message delivered by neurotransmitter molecules. A number of common and very serious neurological problems involve abnormal function of excitatory amino acid synapses. These include epilepsy, several degenerative disorders such as Huntington's disease, and neuronal death following stroke. Unfortunately, there are very few chemical agents which are potent and selective blockers of excitatory amino acid receptors. Na-channel agonists may be used for these purposes.

A drug with high affinity for the receptor could be expected to produce irreversible blockade of synaptic transmission. When labeled with some tracer molecule, such a drug would provide a reliable way of tagging receptors to permit measurement of their number and distribution within cells and tissues. These features would have very valuable consequences for research on excitatory amino acid neurotransmission and for the development of therapeutic agents to treat central nervous system dysfunction in humans and animals. Methods for treating heart and neurological diseases by applying toxins derived from spiders have been described (U.S. Patent No. 4,925,664).

Arthropod animals, including insects, and certain parasitic worms use excitatory amino acids as a major chemical neurotransmitter at their neuromuscular junction and in their central nervous system. Because of the damage done by insect pests and the prevalence of parasitic worm infections in animals and humans in many countries, there is a constant need for potent and specific new pesticides and anthelmintic drugs that are non-toxic to humans, pets, and farm animals.

Chemical insecticides are an integral component of modern agriculture, and are an effective means for reducing crop damage by controlling insect pests. However, chemical agents are under continuous scrutiny due to the potential for environmental contamination, selection of resistant populations of agronomic pests, and toxicity to non-target organisms such as beneficial insects, aquatic organisms, animals and man. As a result, alternative strategies for insect control are being sought that are effective and yet benign to non-target populations and the environment. One of these strategies is to use microorganisms that are naturally occurring pathogens of target insect populations. The expression of scorpion toxins using baculovirus vectors will be an advantage since these toxins have been previously shown to be highly toxic and very specific (Zlotkin et al. (1995) American Chemical Society, Symposium on Agrochemicals).

Due to a combination of problems associated with some synthetic insecticides, including toxicity, environmental hazards, and loss of efficacy due to resistance, there exists a continuing need for the development of novel means of invertebrate control, including the development of genetically engineered recombinant baculoviruses which express protein toxins capable of incapacitating the host more rapidly than the baculovirus infection per se.

Scorpion venoms have been identified as possible sources of compounds providing insecticidal properties. Two insect-selective toxins isolated from the venom of the scorpion *Leiurus* quinquestriatus and affecting sodium conductance have been reported previously (Zlotkin et al. (1985) *Arch. Biochem. Biophys. 240*:877-87). One toxin, AaIT, induced fast excitatory contractive paralysis of fly larvae and the other, LqhIT2, induced slow depressant flaccid paralysis suggesting that these two toxins have different chemical and pharmacological properties (Zlotkin et al. (1971) *Biochimie* (Paris) *53*:1073-1078). Thus, other toxins derived from scorpion venom will also have different chemical and pharmacological properties.

SUMMARY OF THE INVENTION

The present invention relates to isolated polynucleotides comprising a nucleotide sequence encoding a first polypeptide of at least 60 amino acids that has at least 95% identity based on the Clustal method of alignment when compared to a polypeptide selected from the group consisting of a scorpion alpha toxin XIV polypeptide selected from the group consisting of SEQ ID NOs:2, 4, and 6, a scorpion neurotoxin I polypeptide of SEQ ID NO:9, a scorpion depressant toxin LqhIT2 polypeptide selected from ~~the group~~ consisting of SEQ ID NOs:12, 14, and 16. The present invention also relates to an isolated polynucleotide comprising the complement of the nucleotide sequences described above.

It is preferred that the isolated polynucleotides of the claimed invention consist of a nucleic acid sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 8, 11, 13, and 15 that codes for the polypeptide selected from the group consisting of SEQ ID NOs:2, 4, 6, 9, 12, 14, and 16. The present invention also relates to an isolated polynucleotide comprising a nucleotide sequences of at least one of 40 (preferably at least one of 30) contiguous nucleotides derived from a nucleotide sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 8, 11, 13, 15, and the complement of such nucleotide sequences.

The present invention relates to a chimeric gene comprising an isolated polynucleotide of the present invention operably linked to suitable regulatory sequences.

The present invention relates to an isolated host cell comprising a chimeric gene of the present invention or an isolated polynucleotide of the present invention. The host cell may be eukaryotic, such as an insect, a yeast or a plant cell, or prokaryotic, such as a bacterial cell or virus. If the host cell is a virus, it is preferably a baculovirus. It is most preferred that the baculovirus comprises an isolated polynucleotide of the present invention or a chimeric gene of the present invention.

The present invention relates to a process for producing an isolated host cell comprising a chimeric gene of the present invention or an isolated polynucleotide of the present invention, the process comprising either transforming or transfecting an isolated compatible host cell with a chimeric gene or isolated polynucleotide of the present invention.

The present invention relates to a scorpion toxin polypeptide selected from the group of alpha toxin XIV, neurotoxin I, and depressant toxin LqhIT2 of at least 60 amino acids comprising at least 95% homology based on the Clustal method of alignment compared to a polypeptide selected from the group consisting of SEQ ID NOs:2, 4, 6, 9, 12, 14, and 16.

The present invention relates to a method of selecting an isolated polynucleotide that affects the level of expression of a sodium channel agonist polypeptide in a host cell, the method comprising the steps of:

constructing an isolated polynucleotide of the present invention or an isolated chimeric gene of the present invention;

introducing the isolated polynucleotide or the isolated chimeric gene into a host cell;

measuring the level a alpha toxin XIV, a neurotoxin I, or a depressant toxin LqhIT2 polypeptide in the host cell containing the isolated polynucleotide; and

comparing the level of a alpha toxin XIV, a neurotoxin I, or a depressant toxin LqhIT2 polypeptide in the host cell containing the isolated polynucleotide with the level of a alpha toxin XIV, a neurotoxin I, or a depressant toxin LqhIT2 polypeptide in a host cell that does not contain the isolated polynucleotide.

The present invention relates to a method of obtaining a nucleic acid fragment encoding a substantial portion of a alpha toxin XIV, a neurotoxin I, or a depressant toxin LqhIT2 polypeptide gene, preferably a scorpion alpha toxin XIV, a neurotoxin I, or a depressant toxin LqhIT2 polypeptide gene, comprising the steps of: synthesizing an oligonucleotide primer comprising a nucleotide sequence of at least one of 40 (preferably at least one of 30) contiguous nucleotides derived from a nucleotide sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 8, 11, 13, 15, and the complement of such nucleotide sequences; and amplifying a nucleic acid fragment (preferably a cDNA inserted in a cloning vector) using the oligonucleotide primer. The amplified nucleic acid fragment preferably will encode a portion of an alpha toxin XIV, a neurotoxin I, or a depressant toxin LqhIT2 amino acid sequence.

The present invention also relates to a method of obtaining a nucleic acid fragment encoding all or a subsantial portion of the amino acid sequence encoding an alpha toxin XIV, a neurotoxin I, or a depressant toxin LqhIT2 polypeptide comprising the steps of: probing a cDNA or genomic library with an isolated polynucleotide of the present invention; identifying a DNA clone that hybridizes with an isolated polynucleotide of the present invention; isolating the identified DNA clone; and sequencing the cDNA or genomic fragment that comprises the isolated DNA clone.

Another embodiment of the instant invention pertains to a method for expressing a gene encoding a alpha toxin XIV, a neurotoxin I, or a depressant toxin LqhIT2 in the genome of a recombinant baculovirus in insect cell culture or in viable insects wherein said

insect cells or insects have been genetically engineered to express an alpha toxin XIV, a neurotoxin I, or a depressant toxin LqhIT2.

The present invention relates to an expression cassette comprising at least one nucleic acid of Claim 1 operably linked to a promoter.

The present invention relates to a method for positive selection of a transformed cell comprising the steps of transforming a plant cell with a chimeric gene of the present invention or an expression cassette of the present invention; and growing the transformed plant cell under conditions allowing expression of the polynucleotide in an amount sufficient to induce insect resistance to provide a positive selection means.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE LISTING

The invention can be more fully understood from the following detailed description and the accompanying drawings and Sequence Listing which form a part of this application.

Figure 1 shows a comparison of the amino acid sequences of the alpha toxin XIV of the instant invention (SEQ ID NOs:2, 4 and 6) with the sequence of alpha toxin XIV from *Buthus occitanus* (NCBI General Identifier No. 1041278; SEQ ID NO:7). The conserved cysteine residues probably involved in intrachain disulfide bridges are boxed. The first amino acid of the mature toxin is marked by an arrow above the top row.

Figure 2 shows a comparison of the amino acid sequences of the neurotoxin I of the instant invention (SEQ ID NO:9) with the sequence of neurotoxin I from *Buthus occitanus tunetanus* (NCBI General Identifier No. 134335; SEQ ID NO:10). The conserved cysteine residues probably involved in intrachain disulfide bridges are boxed. The first amino acid of the mature toxin is marked by an arrow above the top row.

Figure 3 shows a comparison of the amino acid sequences of the depressant toxin LqhIT2 of the instant invention (SEQ ID NOs:12, 14 and 16) with the sequence of the depressant toxin LqhIT2 from *Leiurus quinquestriatus* (NCBI General Identifier No. 102796; SEQ ID NO:17). The conserved cysteine residues probably involved in intrachain disulfide bridges are boxed. The first amino acid of the mature toxin is marked by an arrow above the top row.

Table 1 lists the polypeptides that are described herein, the designation of the cDNA clones that comprise the nucleic acid fragments encoding polypeptides representing all or a substantial portion of these polypeptides, and the corresponding identifier (SEQ ID NO:) as used in the attached Sequence Listing. The sequence descriptions and Sequence Listing attached hereto comply with the rules governing nucleotide and/or amino acid sequence disclosures in patent applications as set forth in 37 C.F.R. §1.821-1.825.

TABLE 1

Scorpion Sodium Channel Agonists

Protein	Clone Designation	SEQ ID NO: (Nucleotide)	(Amino Acid)
Scorpion Alpha Toxin XIV	lst.pk0004.e12	1	2
Scorpion Alpha Toxin XIV	lst.pk0016.c5.f	3	4
Scorpion Alpha Toxin XIV	lst.pk0015.h11	5	6
Scorpion Neurotoxin I	lst.pk0013.f1	8	9
Scorpion Depressant Toxin LqhIT2	lst.pk0004.c8	11	12
Scorpion Depressant Toxin LqhIT2	lst.pk0013.c9	13	14
Scorpion Depressant Toxin LqhIT2	lst.pkpk0004.e8	15	16

The Sequence Listing contains the one letter code for nucleotide sequence characters and the three letter codes for amino acids as defined in conformity with the IUPAC-IUBMB standards described in *Nucleic Acids Research 13*:3021-3030 (1985) and in the *Biochemical Journal 219 (No. 2)*:345-373 (1984) which are herein incorporated by reference. The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

DETAILED DESCRIPTION OF THE INVENTION

In the context of this disclosure, a number of terms shall be utilized. As used herein, a "polynucleotide" is a nucleotide sequence such as a nucleic acid fragment. A polynucleotide may be a polymer of RNA or DNA that is single- or double-stranded, that optionally contains synthetic, non-natural or altered nucleotide bases. A polynucleotide in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA, or synthetic DNA. An isolated polynucleotide of the present invention may include at least one of 40 contiguous nucleotides, preferably at least one of 30 contiguous nucleotides, most preferably one of at least 15 contiguous nucleotides, of the nucleic acid sequence of the SEQ ID NOs:1, 3, 5, 8, 11, 13 and 15.

"NPV" stands for Nuclear Polyhedrosis Virus, a baculovirus. "Polyhedrosis" refers to any of several virus diseases of insect larvae characterized by dissolution of tissues and accumulation of polyhedral granules in the resultant fluid. "PIBs" are polyhedral inclusion bodies. "AcNPV" stands for the wild-type *Autographa californica* Nuclear Polyhedrosis Virus.

As used herein, "substantially similar" refers to nucleic acid fragments wherein changes in one or more nucleotide bases results in substitution of one or more amino acids, but do not affect the functional properties of the polypeptide encoded by the polynucleotide sequence. "Substantially similar" also refers to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of one or more nucleotides that do not

substantially affect the functional properties of the resulting protein molecule. It is therefore understood that the invention encompasses more than the specific exemplary sequences.

Moreover, substantially similar nucleic acid fragments may also be characterized by their ability to hybridize. Estimates of such homology are provided by either DNA-DNA or DNA-RNA hybridization under conditions of stringency as is well understood by those skilled in the art (Hames and Higgins, Eds. (1985) Nucleic Acid Hybridisation, IRL Press, Oxford, U.K.). Stringency conditions can be adjusted to screen for moderately similar fragments, such as homologous sequences from distantly related organisms, to highly similar fragments, such as genes that duplicate functional enzymes from closely related organisms. Post-hybridization washes determine stringency conditions. One set of preferred conditions uses a series of washes starting with 6X SSC, 0.5% SDS at room temperature for 15 min, then repeated with 2X SSC, 0.5% SDS at 45°C for 30 min, and then repeated twice with 0.2X SSC, 0.5% SDS at 50°C for 30 min. A more preferred set of stringent conditions uses higher temperatures in which the washes are identical to those above except for the temperature of the final two 30 min washes in 0.2X SSC, 0.5% SDS was increased to 60°C. Another preferred set of highly stringent conditions uses two final washes in 0.1X SSC, 0.1% SDS at 65°C.

Substantially similar nucleic acid fragments of the instant invention may also be characterized by the percent identity of the amino acid sequences that they encode to the amino acid sequences disclosed herein, as determined by algorithms commonly employed by those skilled in this art. Suitable nucleic acid fragments (isolated polynucleotides of the present invention) encode polypeptides that are 90% identical to the amino acid sequences reported herein. Most preferred are nucleic acid fragments that encode amino acid sequences that are 95% identical to the amino acid sequences reported herein. Suitable nucleic acid fragments not only have the above homologies but typically encode a polypeptide having at least 50 amino acids, preferably 100 amino acids, more preferably 150 amino acids, still more preferably 200 amino acids, and most preferably 250 amino acids. Sequence alignments and percent identity calculations were performed using the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed using the Clustal method of alignment (Higgins and Sharp (1989) *CABIOS.* 5:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method were KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5.

A "substantial portion" of an amino acid or nucleotide sequence comprises an amino acid or a nucleotide sequence that is sufficient to afford putative identification of the protein or gene that the amino acid or nucleotide sequence comprises. Amino acid and nucleotide sequences can be evaluated either manually by one skilled in the art, or by using computer-

based sequence comparison and identification tools that employ algorithms such as BLAST (Basic Local Alignment Search Tool; Altschul et al. (1993) *J. Mol. Biol. 215*:403-410; see also www.ncbi.nlm.nih.gov/BLAST/). In general, a sequence of ten or more contiguous amino acids or thirty or more contiguous nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene-specific oligonucleotide probes comprising 30 or more contiguous nucleotides may be used in sequence-dependent methods of gene identification (e.g., Southern hybridization) and isolation (e.g., *in situ* hybridization of bacterial colonies or bacteriophage plaques). In addition, short oligonucleotides of 12 or more nucleotides may be used as amplification primers in PCR in order to obtain a particular nucleic acid fragment comprising the primers. Accordingly, a "substantial portion" of a nucleotide sequence comprises a nucleotide sequence that will afford specific identification and/or isolation of a nucleic acid fragment comprising the sequence. The instant specification teaches amino acid and nucleotide sequences encoding polypeptides that comprise one or more particular plant proteins. The skilled artisan, having the benefit of the sequences as reported herein, may now use all or a substantial portion of the disclosed sequences for purposes known to those skilled in this art. Accordingly, the instant invention comprises the complete sequences as reported in the accompanying Sequence Listing, as well as substantial portions of those sequences as defined above.

"Codon degeneracy" refers to divergence in the genetic code permitting variation of the nucleotide sequence without effecting the amino acid sequence of an encoded polypeptide. Accordingly, the instant invention relates to any nucleic acid fragment comprising a nucleotide sequence that encodes all or a substantial portion of the amino acid sequences set forth herein. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a nucleic acid fragment for improved expression in a host cell, it is desirable to design the nucleic acid fragment such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

"Synthetic nucleic acid fragments" can be assembled from oligonucleotide building blocks that are chemically synthesized using procedures known to those skilled in the art. These building blocks are ligated and annealed to form larger nucleic acid fragments which may then be enzymatically assembled to construct the entire desired nucleic acid fragment. "Chemically synthesized", as related to nucleic acid fragment, means that the component nucleotides were assembled *in vitro*. Manual chemical synthesis of nucleic acid fragments may be accomplished using well established procedures, or automated chemical synthesis can be performed using one of a number of commercially available machines. Accordingly, the nucleic acid fragments can be tailored for optimal gene expression based on optimization of nucleotide sequence to reflect the codon bias of the host cell. The skilled artisan

appreciates the likelihood of successful gene expression if codon usage is biased towards those codons favored by the host. Determination of preferred codons can be based on a survey of genes derived from the host cell where sequence information is available.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

"Coding sequence" refers to a nucleotide sequence that codes for a specific amino acid sequence. "Regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

"Promoter" refers to a nucleotide sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. The promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an "enhancer" is a nucleotide sequence which can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic nucleotide segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters which cause a nucleic acid fragment to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". New promoters of various types useful in a variety of cells are constantly being discovered; numerous examples may be found in the compilation by Okamuro and Goldberg (1989) *Biochemistry of Plants 15*:1-82. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not

been completely defined, DNA fragments of different lengths may have identical promoter activity.

The "translation leader sequence" refers to a nucleotide sequence located between the promoter sequence of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency. Examples of translation leader sequences have been described (Turner and Foster (1995) *Mol. Biotechnol. 3*:225-236).

The "3' non-coding sequences" refer to nucleotide sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The use of different 3' non-coding sequences is exemplified by Ingelbrecht et al. (1989) *Plant Cell 1*:671-680.

"RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a ~~perfect~~ complementary copy of the DNA sequence, it is referred to as the primary transcript or it may be a RNA sequence derived from posttranscriptional processing of the primary transcript and is referred to as the mature RNA. "Messenger RNA (mRNA)" refers to the RNA that is without introns and that can be translated into protein by the cell. "cDNA" refers to a double-stranded DNA that is complementary to and derived from mRNA. "Sense" RNA refers to RNA transcript that includes the mRNA and so can be translated into protein by the cell. "Functional RNA" refers to sense RNA, ribozyme RNA, or other RNA that may not be translated but yet has an effect on cellular processes.

The term "operably linked" refers to the association of two or more nucleic acid fragments on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense orientation.

The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of the invention. Expression may also refer to translation of mRNA into a polypeptide. "Overexpression" refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed organisms.

"Altered levels" refers to the production of gene product(s) in transgenic organisms in amounts or proportions that differ from that of normal or non-transformed organisms.

A "signal sequence" is an amino acid sequence that is covalently linked to an amino acid sequence representing a mature protein. The signal sequence directs the protein to the secretory system (Chrispeels (1991) *Ann. Rev. Plant Phys. Plant Mol. Biol. 42*:21-53). "Mature" protein refers to a post-translationally processed polypeptide; i.e., one from which any pre- or propeptides, including signal sequences, present in the primary translation product have been removed. "Precursor" protein refers to the primary product of translation of mRNA; i.e., with pre- and propeptides still present. Pre- and propeptides may be but are not limited to intracellular localization signals.

"Transformation" refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" organisms. Examples of methods of plant transformation include Agrobacterium-mediated transformation (De Blaere et al. (1987) *Meth. Enzymol. 143*:277) and particle-accelerated or "gene gun" transformation technology (Klein et al. (1987) *Nature (London) 327*:70-73; U.S. Patent No. 4,945,050, incorporated herein by reference).

It is understood that "an insect cell" refers to one or more ~~insect~~ cells maintained *in vitro* as well as one or more cells found in an intact, living insect.

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully in Sambrook et al. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, 1989 (hereinafter "Maniatis").

Nucleic acid fragments encoding at least a portion of several scorpion sodium channel agonists have been isolated and identified by comparison of cDNA sequences to public databases containing nucleotide and protein sequences using the BLAST algorithms well known to those skilled in the art. The nucleic acid fragments of the instant invention may be used to isolate cDNAs and genes encoding homologous proteins from the same or other arthropod species. Isolation of homologous genes using sequence-dependent protocols is well known in the art. Examples of sequence-dependent protocols include, but are not limited to, methods of nucleic acid hybridization, and methods of DNA and RNA amplification as exemplified by various uses of nucleic acid amplification technologies (e.g., polymerase chain reaction, ligase chain reaction).

For example, genes encoding other alpha toxin XIV, either as cDNAs or genomic DNAs, could be isolated directly by using all or a portion of the instant nucleic acid fragments as DNA hybridization probes to screen libraries from any desired arthropod employing methodology well known to those skilled in the art. Specific oligonucleotide probes based upon the instant nucleic acid sequences can be designed and synthesized by methods known in the art (Maniatis). Moreover, the entire sequences can be used directly to synthesize DNA probes by methods known to the skilled artisan such as random primer

DNA labeling, nick translation, or end-labeling techniques, or RNA probes using available *in vitro* transcription systems. In addition, specific primers can be designed and used to amplify a part or all of the instant sequences. The resulting amplification products can be labeled directly during amplification reactions or labeled after amplification reactions, and used as probes to isolate full length cDNA or genomic fragments under conditions of appropriate stringency.

In addition, two short segments of the instant nucleic acid fragments may be used in polymerase chain reaction protocols to amplify longer nucleic acid fragments encoding homologous genes from DNA or RNA. The polymerase chain reaction may also be performed on a library of cloned nucleic acid fragments wherein the sequence of one primer is derived from the instant nucleic acid fragments, and the sequence of the other primer takes advantage of the presence of the polyadenylic acid tracts to the 3' end of the mRNA precursor encoding arthropod genes. Alternatively, the second primer sequence may be based upon sequences derived from the cloning vector. For example, the skilled artisan can follow the RACE protocol (Frohman et al. (1988) *Proc. Natl. Acad. Sci. USA 85*:8998-9002) to generate cDNAs by using PCR to amplify copies of the region between a single point in the transcript and the 3' or 5' end. Primers oriented in the 3' and 5' directions can be designed from the instant sequences. Using commercially available 3' RACE or 5' RACE systems (BRL), specific 3' or 5' cDNA fragments can be isolated (Ohara et al. (1989) *Proc. Natl. Acad. Sci. USA 86*:5673-5677; Loh et al. (1989) *Science 243*:217-220). Products generated by the 3' and 5' RACE procedures can be combined to generate full-length cDNAs (Frohman and Martin (1989) *Techniques 1*:165).

Availability of the instant nucleotide and deduced amino acid sequences facilitates immunological screening of cDNA expression libraries. Synthetic peptides representing portions of the instant amino acid sequences may be synthesized. These peptides can be used to immunize animals to produce polyclonal or monoclonal antibodies with specificity for peptides or proteins comprising the amino acid sequences. These antibodies can be then be used to screen cDNA expression libraries to isolate full-length cDNA clones of interest (Lerner (1984) *Adv. Immunol. 36*:1-34; Maniatis).

The nucleic acid fragments of the instant invention may be used to create transgenic plants in which the disclosed scorpion sodium channel agonists are expressed. This would be useful as a means for controlling insect pests by producing plants that are more insect-tolerant than the naturally occurring variety.

Expression in plants of the proteins of the instant invention may be accomplished by first constructing a chimeric gene in which the coding region is operably linked to a promoter capable of directing expression of a gene in the desired tissues at the desired stage of development. For reasons of convenience, the chimeric gene may comprise promoter sequences and translation leader sequences derived from the same genes. 3' Non-coding

sequences encoding transcription termination signals may also be provided. The instant chimeric gene may also comprise one or more introns in order to facilitate gene expression.

Plasmid vectors comprising the instant chimeric gene can then constructed. The choice of plasmid vector is dependent upon the method that will be used to transform host plants. The skilled artisan is well aware of the genetic elements that must be present on the plasmid vector in order to successfully transform, select and propagate host cells containing the chimeric gene. The skilled artisan will also recognize that different independent transformation events will result in different levels and patterns of expression (Jones et al. (1985) *EMBO J. 4*:2411-2418; De Almeida et al. (1989) *Mol. Gen. Genetics 218*:78-86), and thus that multiple events must be screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of DNA, Northern analysis of mRNA expression, Western analysis of protein expression, LC-MS, or phenotypic analysis.

The instant polypeptides (or portions thereof) may be produced in heterologous host cells, particularly in the cells of microbial hosts, and can be used to prepare antibodies to the these proteins by methods well known to those skilled in the art. The antibodies are useful for detecting the polypeptides of the instant invention *in situ* in cells or *in vitro* in cell extracts. Preferred heterologous host cells for production of the instant polypeptides are microbial hosts. Microbial expression systems and expression vectors containing regulatory sequences that direct high level expression of foreign proteins are well known to those skilled in the art. Any of these could be used to construct a chimeric gene for production of the instant polypeptides. This chimeric gene could then be introduced into appropriate microorganisms via transformation to provide high level expression of the encoded scorpion sodium channel agonist. An example of a vector for high level expression of the instant polypeptides in a bacterial host is provided (Example 8).

Insecticidal baculoviruses have great potential to provide an environmentally benign method for agricultural insect pest control. However, improvements to efficacy are required in order to make these agents competitive with current chemical pest control agents. One approach for making such improvements is through genetic alteration of the virus. For instance, it may be possible to modify the viral genome in order to improve the host range of the virus, to increase the environmental stability and persistence of the virus, or to improve the infectivity and transmission of the virus. In addition, improving the rate at which the virus acts to compromise the infected insect would significantly enhance the attractiveness of insecticidal baculoviruses as adjuncts or replacements for chemical pest control agents. One method for increasing the speed with which the virus affects its insect host is to introduce into the baculovirus foreign genes that encode proteins that are toxic to the insect wherein death or incapacitation of the insect is no longer dependent solely on the course of the viral

infection, but instead is aided by the accumulation of toxic levels of the foreign protein. The results are insecticidal recombinant baculoviruses.

Recombinant baculoviruses expressing the instant scorpion sodium channel agonists (or portions thereof) may be prepared by protocols now known to the art (e.g., Tomalski et al., U.S. Patent No. 5,266,317, exemplifying neurotoxins from the insect-parasitic mites; McCutchen et al. (1991) *Bio/Technology 9*:848-852; Maeda et al. (1991) *Virology 184*:777-780, illustrating construction of a recombinant baculovirus expressing AalT; also see O'Reilly et al. (1992) *Baculovirus Expression Vectors: A Laboratory Manual*, W. H. Freeman and Company, New York; King and Possee (1992) *The Baculovirus Expression System*, Chapman and Hall, London; U.S. Patent No. 4,745,051). These methods of gene expression provide economical preparation of foreign proteins in a eukaryotic expression vector system, in many instances yielding proteins that have achieved their proper tertiary conformation and formed the proper disulfide bridges necessary for activity.

Commonly, the introduction of heterologous genes into the baculovirus genome occurs by homologous recombination between viral genomic DNA and a suitable "transfer vector" containing the heterologous gene of interest. These transfer vectors are generally plasmid DNAs that are capable of autonomous replication in bacterial hosts, affording facile genetic manipulation. Baculovirus transfer vectors also contain a genetic cassette comprising a region of the viral genome that has been modified to include the following features (listed in the 5' to 3' direction): 1) viral DNA comprising the 5' region of a non-essential genomic region; 2) a viral promoter; 3) one or more DNA sequences encoding restriction enzyme sites facilitating insertion of heterologous DNA sequences; 4) a transcriptional termination sequence; and 5) viral DNA comprising the 3' region of a non-essential genomic region. A heterologous gene of interest is inserted into the transfer vector at the restriction site downstream of the viral promoter. The resulting cassette comprises a chimeric gene wherein the heterologous gene is under the transcriptional control of the viral promoter and transcription termination sequences present on the transfer vector. Moreover, this chimeric gene is flanked by viral DNA sequences that facilitate homologous recombination at a non-essential region of the viral genome. Recombinant viruses are created by co-transfecting insect cells that are capable of supporting viral replication with viral genomic DNA and the recombinant transfer vector. Homologous recombination between the flanking viral DNA sequences present on the transfer vector and the homologous sequences on the viral genomic DNA takes place and results in insertion of the chimeric gene into a region of the viral genome that does not disrupt an essential viral function. The infectious recombinant virion consists of the recombined genomic DNA, referred to as the baculovirus expression vector, surrounded by a protein coat.

In a preferred embodiment, the non-essential region of the viral genome that is present on the transfer vector comprises the region of the viral DNA responsible for polyhedrin

production. Most preferred is a transfer vector that contains the entire polyhedrin gene between the flanking sequences that are involved in homologous recombination. Recombination with genomic DNA from viruses that are defective in polyhedrin production (due to a defect in the genomic copy of the polyhedrin gene) will result in restoration of the polyhedrin-positive phenotype. This strategy facilitates identification and selection of recombinant viruses.

In another embodiment, baculoviral genomic DNA can be directly modified by introduction of a unique restriction enzyme recognition sequence into a non-essential region of the viral genome. A chimeric gene comprising the heterologous gene to be expressed by the recombinant virus and operably linked to regulatory sequences capable of directing gene expression in baculovirus-infected insect cells, can be constructed and inserted directly into the viral genome at the unique restriction site. This strategy eliminates both the need for construction of transfer vectors and reliance on homologous recombination for generation of recombinant viruses. This technology is described by Ernst et al. (Ernst et al. (1994) *Nuc. Acid Res. 22*: 2855-2856), and in WO94/28114.

Recombinant baculovirus expression vectors suitable for delivering genetically encoded insect-specific neurotoxins require optimal toxin gene expression for maximum efficacy. A number of strategies can be used by the skilled artisan to design and prepare recombinant baculoviruses wherein toxin gene expression results in sufficient quantities of toxin produced at appropriate times during infection in a functional form and available for binding to target cells within the insect host.

The isolated toxin gene fragment may be digested with appropriate enzymes and may be inserted into the pTZ-18R plasmid (Pharmacia, Piscataway, NJ) at the multiple cloning site using standard molecular cloning techniques. Following transformation of *E. coli* DH5αMCR, isolated colonies may be chosen and plasmid DNA prepared. Positive clones will be identified and sequenced with the commercially available forward and reverse primers.

Spodoptera frugiperda cells (Sf-9) may be propagated in ExCell® 401 media (JRH Biosciences, Lenexa, KS) supplemented with 3.0% fetal bovine serum. Lipofectin® (50 µL at 0.1 mg/mL, Gibco/BRL) may be added to a 50 µL aliquot of the transfer vector containing the toxin gene of interest (500 ng) and linearized polyhedrin-negative AcNPV (2.5 µg, Baculogold® viral DNA, Pharmigen, San Diego, CA). Sf-9 cells (approximate 50% monolayer) may be co-transfected with the viral DNA/transfer vector solution. The supernatant fluid from the co-transfection experiment may be collected at 5 days post-transfection and recombinant viruses may be isolated employing standard plaque purification protocols, wherein only polyhedrin-positive plaques will be selected (Granados, R. R., Lawler, K. A., *Virology* (1981), *108*, 297-308).

To propagate the recombinant virus of interest, isolated plaques may be picked and suspended in 500 μL of ExCell® media supplemented with 2.5% fetal bovine serum. Sf-9 cells in 35 mM petri dishes (50% monolayer) may be inoculated with 100 μL of the viral suspension, and supernatant fluids collected at 5 days post infection. These supernatant fluids will be used to inoculate cultures for large scale propagation of recombinant viruses.

Expression of the encoded toxin gene by the recombinant baculovirus will be confirmed using a bioassay, LCMS, or antibodies. The presence of toxin activity in the recombinant viruses will be monitored *in vivo*. These assays involve comparison of biological activity of recombinant viruses to wild-type. Third instar larvae of *H. virescens* are infected orally by consumption of diet that contains test and control viruses and the larvae monitored for behavioral changes and mortality.

Isolated plugs of a standard insect diet are inoculated with approximately 5000 PIBs of each virus. Individual larvae that have not fed for 12 h prior to beginning of the bioassay are allowed to consume the diet for 24 h. The larvae are transferred to individual wells in a diet tray and monitored for symptoms and mortality on a daily basis (Zlotkin et al. (1991) *Biochimie (Paris) 53*:1073-1078).

EXAMPLES

The present invention is further defined in the following Examples, in which all parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

EXAMPLE 1

Composition of cDNA Libraries; Isolation and Sequencing of cDNA Clones

cDNA libraries representing mRNAs from various *Leiurus* scorpion telson tissues were prepared. cDNA libraries may be prepared by any one of many methods available. For example, the cDNAs may be introduced into plasmid vectors by first preparing the cDNA libraries in Uni-ZAP™ XR vectors according to the manufacturer's protocol (Stratagene Cloning Systems, La Jolla, CA). The Uni-ZAP™ XR libraries are converted into plasmid libraries according to the protocol provided by Stratagene. Upon conversion, cDNA inserts will be contained in the plasmid vector pBluescript. In addition, the cDNAs may be introduced directly into precut Bluescript II SK(+) vectors (Stratagene) using T4 DNA ligase (New England Biolabs), followed by transfection into DH10B cells according to the manufacturer's protocol (GIBCO BRL Products). Once the cDNA inserts are in plasmid vectors, plasmid DNAs are prepared from randomly picked bacterial colonies containing recombinant pBluescript plasmids, or the insert cDNA sequences are amplified via

polymerase chain reaction using primers specific for vector sequences flanking the inserted cDNA sequences. Amplified insert DNAs or plasmid DNAs are sequenced in dye-primer sequencing reactions to generate partial cDNA sequences (expressed sequence tags or "ESTs"; see Adams et al., (1991) *Science 252*:1651-1656). The resulting ESTs are analyzed using a Perkin Elmer Model 377 fluorescent sequencer.

EXAMPLE 2

Identification of cDNA Clones

ESTs encoding scorpion sodium channel agonists were identified by conducting BLAST (Basic Local Alignment Search Tool; Altschul et al. (1993) *J. Mol. Biol. 215*:403-410; see also www.ncbi.nlm.nih.gov/BLAST/) searches for similarity to sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein Data Bank, the last major release of the SWISS-PROT protein sequence database, EMBL, and DDBJ databases). The cDNA sequences obtained in Example 1 were analyzed for similarity to all publicly available DNA sequences contained in the "nr" database using the BLASTN algorithm provided by the National Center for Biotechnology Information (NCBI). The DNA sequences were translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the "nr" database using the BLASTX algorithm (Gish and States (1993) *Nat. Genet. 3*:266-272) provided by the NCBI. For convenience, the P-value (probability) of observing a match of a cDNA sequence to a sequence contained in the searched databases merely by chance as calculated by BLAST are reported herein as "pLog" values, which represent the negative of the logarithm of the reported P-value. Accordingly, the greater the pLog value, the greater the likelihood that the cDNA sequence and the BLAST "hit" represent homologous proteins.

EXAMPLE 3

Characterization of cDNA Clones Encoding Alpha Toxin XIV

The BLASTX search using the EST sequences from clones listed in Table 3 revealed similarity of the polypeptides encoded by the cDNAs to alpha toxin XIV from *Buthus occitanus* (NCBI General Identifier No. 1041278). Shown in Table 3 are the BLASTP results for individual ESTs:

TABLE 3

BLAST Results for Sequences Encoding Polypeptides Homologous to Alpha Toxin XIV

Clone	BLAST pLog Score 1041278
lst.pk0004.e12	24.15
lst.pk0016.c5.f	28.00
lst.pk0015.h11.f	29.70

The nucleotide sequences from the clones presented above encode entire toxins and all or part of the corresponding signal sequence. The amino acid sequence set forth in SEQ ID NO:2 contains a signal sequence (amino acids 1-11) and a mature toxin (amino acids 12-75). The amino acid sequence set forth in SEQ ID NO:4 contains a signal sequence (amino acids 1-12) and a mature toxin (amino acids 13-79). The amino acid sequence set forth in SEQ ID NO:6 contains a signal sequence (amino 1-19) and a mature toxin (amino acids 20-87).

Figure 1 presents an alignment of the amino acid sequences set forth in SEQ ID NOs:2, 4 and 6 and the *Buthus occitanus* sequence (NCBI General Identifier No. 1041278). The data in Table 4 represents a calculation of the percent identity of the amino acid sequences set forth in SEQ ID NOs:2, 4 and 6 and the *Buthus occitanus* sequence (SEQ ID NO:7).

TABLE 4

Percent Identity of Amino Acid Sequences Deduced From the Nucleotide Sequences of cDNA Clones Encoding Polypeptides Homologous to Alpha Toxin XIV

SEQ ID NO.	Percent Identity to 1041278
2	60.0
4	68.4
6	64.7

Sequence alignments and percent identity calculations were performed using the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed using the Clustal method of alignment (Higgins and Sharp (1989) *CABIOS. 5*:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method were KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. Sequence alignments and BLAST scores and probabilities indicate that the instant nucleic acid fragments encode three distinct, entire, scorpion alpha toxin XIV, two of which have partial signal sequences and one has the entire signal sequence.

EXAMPLE 4

Characterization of cDNA Clones Encoding Neruotoxin I

The BLASTX search using the EST sequence from clone lst.pk0013.f1 revealed similarity of the protein encoded by the cDNAs to neurotoxin I from *Buthus occitanus tunetanus* (NCBI General Identifier No. 134335), with a pLog value of 36.70. The amino acid sequence set forth in SEQ ID NO:9 contains a signal sequence (amino acids 1-19) and a mature protein (amino acids 20-84). Figure 2 presents an alignment of the amino acid sequences set forth in SEQ ID NO:9 and the *Buthus occitanus* sequence (SEQ ID NO:10).

The amino acid sequence presented in SEQ ID NO:9 is 80.0% identical to the *Buthus occitanus* sequence.

Sequence alignments and percent identity calculations were performed using the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed using the Clustal method of alignment (Higgins and Sharp (1989) *CABIOS. 5*:151-153) with the default parameters (KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5). Sequence alignments and BLAST scores and probabilities indicate that the instant nucleic acid fragment encodes an entire scorpion neurotoxin I with its signal sequence.

EXAMPLE 5

Characterization of cDNA Clones Encoding Depressant Toxin LqhIT2

The BLASTX search using the EST sequences from clones listed in Table 5 revealed similarity of the polypeptides encoded by the cDNAs to depressant toxin LqhIT2 from *Leiurus quinquestriatus* (NCBI General Identifier No. 102796). Shown in Table 5 are the BLAST results for individual ESTs:

TABLE 5

BLAST Results for Sequences Encoding Polypeptides Homologous to Depressant Toxin LQHIT2

Clone	BLAST pLog Score 102796
lst.pk0004.c8	39.75
lst.pk0013.c9	32.40
lst.pkpk0004.e8	18.85

The nucleotide sequences from the clones presented above encode entire toxins and all or part of the corresponding signal sequence. The amino acid sequence set forth in SEQ ID NO:12 contains a signal sequence (amino acids 1-21) and a mature protein (amino acids 22-85). The amino acid sequence set forth in SEQ ID NO:14 contains a signal sequence (amino acids 1-21) and a mature protein (amino acids 22-85). The amino acid sequence set forth in SEQ ID NO:6 contains a signal sequence (amino acids 1-19) and a mature protein (amino acids 20-85).

Figure 3 presents an alignment of the amino acid sequences set forth in SEQ ID NOs:12, 14 and 16 and the *Leiurus quinquestriatus* sequence (NCBI General Identifier No. 102796). The data in Table 6 represents a calculation of the percent identity of the amino acid sequences set forth in SEQ ID NOs:12, 14 and 16 and the *Leiurus quinquestriatus* sequence (SEQ ID NO:17).

TABLE 6

Percent Identity of Amino Acid Sequences Deduced From the Nucleotide Sequences of cDNA Clones Encoding Polypeptides Homologous to Depressant Toxin LqhIT2

SEQ ID NO.	Percent Identity to 102796
12	86.9
14	72.1
16	45.9

Sequence alignments and percent identity calculations were performed using the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed using the Clustal method of alignment (Higgins and Sharp (1989) *CABIOS. 5*:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method were KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. Sequence alignments and BLAST scores and probabilities indicate that the instant nucleic acid fragments encode three distinct entire scorpion depressant toxin LqhIT2 proteins with their signal sequences.

EXAMPLE 6

Expression of Chimeric Genes in Monocot Cells

A chimeric gene comprising a cDNA encoding the instant polypeptides in sense orientation with respect to the maize 27 kD zein promoter that is located 5' to the cDNA fragment, and the 10 kD zein 3' end that is located 3' to the cDNA fragment, can be constructed. The cDNA fragment of this gene may be generated by polymerase chain reaction (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites (Nco I or Sma I) can be incorporated into the oligonucleotides to provide proper orientation of the DNA fragment when inserted into the digested vector pML103 as described below. Amplification is then performed in a standard PCR. The amplified DNA is then digested with restriction enzymes Nco I and Sma I and fractionated on an agarose gel. The appropriate band can be isolated from the gel and combined with a 4.9 kb Nco I-Sma I fragment of the plasmid pML103. Plasmid pML103 has been deposited under the terms of the Budapest Treaty at ATCC (American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209), and bears accession number ATCC 97366. The DNA segment from pML103 contains a 1.05 kb Sal I-Nco I promoter fragment of the maize 27 kD zein gene and a 0.96 kb Sma I-Sal I fragment from the 3' end of the maize 10 kD zein gene in the vector pGem9Zf(+) (Promega). Vector and insert DNA can be ligated at 15°C overnight, essentially as described (Maniatis). The ligated DNA may then be used to transform *E. coli* XL1-Blue (Epicurian Coli XL-1 Blue™; Stratagene). Bacterial transformants can be screened by restriction enzyme digestion of plasmid DNA and limited nucleotide sequence

analysis using the dideoxy chain termination method (Sequenase™ DNA Sequencing Kit; U.S. Biochemical). The resulting plasmid construct would comprise a chimeric gene encoding, in the 5' to 3' direction, the maize 27 kD zein promoter, a cDNA fragment encoding the instant polypeptides, and the 10 kD zein 3' region.

The chimeric gene described above can then be introduced into corn cells by the following procedure. Immature corn embryos can be dissected from developing caryopses derived from crosses of the inbred corn lines H99 and LH132. The embryos are isolated 10 to 11 days after pollination when they are 1.0 to 1.5 mm long. The embryos are then placed with the axis-side facing down and in contact with agarose-solidified N6 medium (Chu et al., (1975) *Sci. Sin. Peking* 18:659-668). The embryos are kept in the dark at 27°C. Friable embryogenic callus consisting of undifferentiated masses of cells with somatic proembryoids and embryoids borne on suspensor structures proliferates from the scutellum of these immature embryos. The embryogenic callus isolated from the primary explant can be cultured on N6 medium and sub-cultured on this medium every 2 to 3 weeks.

The plasmid, p35S/Ac (obtained from Dr. Peter Eckes, Hoechst Ag, Frankfurt, Germany) may be used in transformation experiments in order to provide for a selectable marker. This plasmid contains the *Pat* gene (see European Patent Publication 0 242 236) which encodes phosphinothricin acetyl transferase (PAT). The enzyme PAT confers resistance to herbicidal glutamine synthetase inhibitors such as phosphinothricin. The *pat* gene in p35S/Ac is under the control of the 35S promoter from cauliflower mosaic virus (Odell et al. (1985) *Nature* 313:810-812) and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*.

The particle bombardment method (Klein et al. (1987) *Nature* 327:70-73) may be used to transfer genes to the callus culture cells. According to this method, gold particles (1 μm in diameter) are coated with DNA using the following technique. Ten μg of plasmid DNAs are added to 50 μL of a suspension of gold particles (60 mg per mL). Calcium chloride (50 μL of a 2.5 M solution) and spermidine free base (20 μL of a 1.0 M solution) are added to the particles. The suspension is vortexed during the addition of these solutions. After 10 minutes, the tubes are briefly centrifuged (5 sec at 15,000 rpm) and the supernatant removed. The particles are resuspended in 200 μL of absolute ethanol, centrifuged again and the supernatant removed. The ethanol rinse is performed again and the particles resuspended in a final volume of 30 μL of ethanol. An aliquot (5 μL) of the DNA-coated gold particles can be placed in the center of a Kapton™ flying disc (Bio-Rad Labs). The particles are then accelerated into the corn tissue with a Biolistic™ PDS-1000/He (Bio-Rad Instruments, Hercules CA), using a helium pressure of 1000 psi, a gap distance of 0.5 cm and a flying distance of 1.0 cm.

For bombardment, the embryogenic tissue is placed on filter paper over agarose-solidified N6 medium. The tissue is arranged as a thin lawn and covered a circular area of

about 5 cm in diameter. The petri dish containing the tissue can be placed in the chamber of the PDS-1000/He approximately 8 cm from the stopping screen. The air in the chamber is then evacuated to a vacuum of 28 inches of Hg. The macrocarrier is accelerated with a helium shock wave using a rupture membrane that bursts when the He pressure in the shock tube reaches 1000 psi.

Seven days after bombardment the tissue can be transferred to N6 medium that contains gluphosinate (2 mg per liter) and lacks casein or proline. The tissue continues to grow slowly on this medium. After an additional 2 weeks the tissue can be transferred to fresh N6 medium containing gluphosinate. After 6 weeks, areas of about 1 cm in diameter of actively growing callus can be identified on some of the plates containing the glufosinate-supplemented medium. These calli may continue to grow when sub-cultured on the selective medium.

Plants can be regenerated from the transgenic callus by first transferring clusters of tissue to N6 medium supplemented with 0.2 mg per liter of 2,4-D. After two weeks the tissue can be transferred to regeneration medium (Fromm et al., (1990) *Bio/Technology* *8*:833-839).

EXAMPLE 7

Expression of Chimeric Genes in Dicot Cells

A seed-specific expression cassette composed of the promoter and transcription terminator from the gene encoding the β subunit of the seed storage protein phaseolin from the bean *Phaseolus vulgaris* (Doyle et al. (1986) *J. Biol. Chem.* *261*:9228-9238) can be used for expression of the instant polypeptides in transformed soybean. The phaseolin cassette includes about 500 nucleotides upstream (5') from the translation initiation codon and about 1650 nucleotides downstream (3') from the translation stop codon of phaseolin. Between the 5' and 3' regions are the unique restriction endonuclease sites Nco I (which includes the ATG translation initiation codon), Sma I, Kpn I and Xba I. The entire cassette is flanked by Hind III sites.

The cDNA fragment of this gene may be generated by polymerase chain reaction (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites can be incorporated into the oligonucleotides to provide proper orientation of the DNA fragment when inserted into the expression vector. Amplification is then performed as described above, and the isolated fragment is inserted into a pUC18 vector carrying the seed expression cassette.

Soybean embryos may then be transformed with the expression vector comprising sequences encoding the instant polypeptides. To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface sterilized, immature seeds of the soybean cultivar A2872, can be cultured in the light or dark at 26°C on an appropriate agar medium for 6-10 weeks. Somatic embryos which produce secondary embryos are then excised and

placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos which multiplied as early, globular staged embryos, the suspensions are maintained as described below.

Soybean embryogenic suspension cultures can maintained in 35 mL liquid media on a rotary shaker, 150 rpm, at 26°C with florescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 mL of liquid medium.

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein et al. (1987) *Nature* (London) *327*:70, U.S. Patent No. 4,945,050). A DuPont Biolistic™ PDS1000/HE instrument (helium retrofit) can be used for these transformations.

A selectable marker gene which can be used to facilitate soybean transformation is a chimeric gene composed of the 35S promoter from cauliflower mosaic virus (Odell et al. (1985) *Nature 313*:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz et al.(1983) *Gene 25*:179-188) and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The seed expression cassette comprising the phaseolin 5' region, the fragment encoding the instant polypeptides and the phaseolin 3' region can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

To 50 µL of a 60 mg/mL 1 µm gold particle suspension is added (in order): 5 µL DNA (1 µg/µL), 20 µl spermidine (0.1 M), and 50 µL $CaCl_2$ (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400 µL 70% ethanol and resuspended in 40 µL of anhydrous ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five µL of the DNA-coated gold particles are then loaded on each macro carrier disk.

Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5-10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as described above.

Five to seven days post bombardment, the liquid media may be exchanged with fresh media, and eleven to twelve days post bombardment with fresh media containing 50 mg/mL hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post bombardment, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into

individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

EXAMPLE 8

Expression of Chimeric Genes in Microbial Cells

The cDNAs encoding the instant polypeptides can be inserted into the T7 *E. coli* expression vector pBT430. This vector is a derivative of pET-3a (Rosenberg et al. (1987) *Gene 56*:125-135) which employs the bacteriophage T7 RNA polymerase/T7 promoter system. Plasmid pBT430 was constructed by first destroying the EcoR I and Hind III sites in pET-3a at their original positions. An oligonucleotide adaptor containing EcoR I and Hind III sites was inserted at the BamH I site of pET-3a. This created pET-3aM with additional unique cloning sites for insertion of genes into the expression vector. Then, the Nde I site at the position of translation initiation was converted to an Nco I site using oligonucleotide-directed mutagenesis. The DNA sequence of pET-3aM in this region, 5'-CATATGG, was converted to 5'-CCCATGG in pBT430.

Plasmid DNA containing a cDNA may be appropriately digested to release a nucleic acid fragment encoding the protein. This fragment may then be purified on a 1% NuSieve GTG™ low melting agarose gel (FMC). Buffer and agarose contain 10 μg/ml ethidium bromide for visualization of the DNA fragment. The fragment can then be purified from the agarose gel by digestion with GELase™ (Epicentre Technologies) according to the manufacturer's instructions, ethanol precipitated, dried and resuspended in 20 μL of water. Appropriate oligonucleotide adapters may be ligated to the fragment using T4 DNA ligase (New England Biolabs, Beverly, MA). The fragment containing the ligated adapters can be purified from the excess adapters using low melting agarose as described above. The vector pBT430 is digested, dephosphorylated with alkaline phosphatase (NEB) and deproteinized with phenol/chloroform as described above. The prepared vector pBT430 and fragment can then be ligated at 16°C for 15 hours followed by transformation into DH5 electrocompetent cells (GIBCO BRL). Transformants can be selected on agar plates containing LB media and 100 μg/mL ampicillin. Transformants containing the gene encoding the instant polypeptides are then screened for the correct orientation with respect to the T7 promoter by restriction enzyme analysis.

For high level expression, a plasmid clone with the cDNA insert in the correct orientation relative to the T7 promoter can be transformed into *E. coli* strain BL21(DE3) (Studier et al. (1986) *J. Mol. Biol. 189*:113-130). Cultures are grown in LB medium containing ampicillin (100 mg/L) at 25°C. At an optical density at 600 nm of approximately 1, IPTG (isopropylthio-β-galactoside, the inducer) can be added to a final concentration of 0.4 mM and incubation can be continued for 3 h at 25°. Cells are then harvested by

centrifugation and re-suspended in 50 µL of 50 mM Tris-HCl at pH 8.0 containing 0.1 mM DTT and 0.2 mM phenyl methylsulfonyl fluoride. A small amount of 1 mm glass beads can be added and the mixture sonicated 3 times for about 5 seconds each time with a microprobe sonicator. The mixture is centrifuged and the protein concentration of the supernatant determined. One µg of protein from the soluble fraction of the culture can be separated by SDS-polyacrylamide gel electrophoresis. Gels can be observed for protein bands migrating at the expected molecular weight.

EXAMPLE 9

Expression of Chimeric Genes in Insect Cells

The cDNAs encoding the instant polypeptides may be introduced into the baculovirus genome itself. For this purpose the cDNAs may be placed under the control of the polyhedron promoter, the IE1 promoter, or any other one of the baculovirus promoters. The cDNA, together with appropriate leader sequences is then inserted into a baculovirus transfer vector using standard molecular cloning techniques. Following transformation of *E. coli* DH5α, isolated colonies are chosen and plasmid DNA is prepared and is analyzed by restriction enzyme analysis. Colonies containing the appropriate fragment are isolated, propagated, and plasmid DNA is prepared for cotransfection.

Spodoptera frugiperda cells (Sf-9) are propagated in ExCell® 401 media (JRH Biosciences, Lenexa, KS) supplemented with 3.0% fetal bovine serum. Lipofectin® (50 µL at 0.1 mg/mL, Gibco/BRL) is added to a 50 µL aliquot of the transfer vector containing the toxin gene (500 ng) and linearized polyhedrin-negative AcNPV (2.5 µg, Baculogold® viral DNA, Pharmigen, San Diego, CA). Sf-9 cells (approximate 50% monolayer) are co-transfected with the viral DNA/transfer vector solution. The supernatant fluid from the co-transfection experiment is collected at 5 days post-transfection and recombinant viruses are isolated employing standard plaque purification protocols, wherein only polyhedrin-positive plaques are selected (O'Reilly et al. (1992), *Baculovirus Expression Vectors: A Laboratory Manual*, W. H. Freeman and Company, New York.). Sf-9 cells in 35 mM petri dishes (50% monolayer) are inoculated with 100 µL of a serial dilution of the viral suspension, and supernatant fluids are collected at 5 days post infection. In order to prepare larger quantities of virus for characterization, these supernatant fluids are used to inoculate larger tissue cultures for large scale propagation of recombinant viruses. Expression of the instant polypeptides encoded by the recombinant baculovirus is confirmed by bioassay.

CLAIMS

What is claimed is:

1. A composition comprising an isolated polynucleotide comprising a nucleotide sequence encoding a first polypeptide of at least 60 amino acids that has at least 95% identity based on the Clustal method of alignment when compared to a polypeptide selected from the group consisting of a polypeptide of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:14, and SEQ ID NO:16,

or an isolated polynucleotide comprising the complement of the nucleotide sequence.

2. The composition of Claim 1, wherein the isolated nucleotide sequence consists of a nucleic acid sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 8, 11, 13, and 15 that codes for the polypeptide selected from the group consisting of SEQ ID NOs:2, 4, 6, 9, 12, 14, and 16.

3. The isolated nucleic acid fragment of Claim 1 wherein the nucleotide sequence of the fragment encodes a mature protein.

4. The composition of Claim 1 wherein the isolated polynucleotide is DNA.

5. The composition of Claim 1 wherein the isolated polynucleotide is RNA.

6. A chimeric gene comprising the isolated polynucleotide of Claim 1 operably linked to suitable regulatory sequences.

7. An isolated host cell comprising the chimeric gene of Claim 6.

8. An isolated host cell comprising an isolated polynucleotide of Claim 1, Claim 3 or Claim 4.

9. The isolated host cell of Claim 7 wherein the isolated host selected from the group consisting of yeast, insect, bacteria, plant, and virus.

10. A virus comprising the isolated polynucleotide of Claim 1.

11. A composition comprising a polypeptide of at least 60 amino acids that has at least 95% identity based on the Clustal method of alignment when compared to a polypeptide selected from the group consisting of a polypeptide of SEQ ID NOs:2, 4, 6, 9, 12, 14, and 16.

12. A method of selecting an isolated polynucleotide that affects the level of expression of a polypeptide in a plant cell, the method comprising the steps of:

(a) constructing an isolated polynucleotide comprising a nucleotide sequence of at least one of 30 contiguous nucleotides derived from the isolated polynucleotide of Claim 1;

(b) introducing the isolated polynucleotide into a plant cell;

(c) measuring the level of a polypeptide in the plant cell containing the polynucleotide; and

(d) comparing the level of polypeptide in the plant cell containing the isolated polynucleotide with the level of polypeptide in a plant cell that does not contain the isolated polynucleotide.

13. The method of Claim 12 wherein the isolated polynucleotide consists of a nucleotide sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 8, 11, 13, and 15 that codes for the polypeptide selected from the group consisting of SEQ ID NOs:2, 4, 6, 9, 12, 14, and 16.

14. A method of selecting an isolated polynucleotide that affects the level of expression of polypeptide in a plant cell, the method comprising the steps of:

(a) constructing an isolated polynucleotide of Claim 1;

(b) introducing the isolated polynucleotide into a plant cell;

(c) measuring the level of polypeptide in the plant cell containing the polynucleotide; and

(d) comparing the level of polypeptide in the plant cell containing the isolated polynucleotide with the level of polypeptide in a plant cell that does not contain the polynucleotide.

15.A method of obtaining a nucleic acid fragment encoding a polypeptide comprising the steps of:

(a) synthesizing an oligonucleotide primer comprising a nucleotide sequence of at least one of 30 contiguous nucleotides derived from a nucleotide sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 8, 11, 13, 15, and the complement of such nucleotide sequences; and

(b) amplifying a nucleic acid sequence using the oligonucleotide primer.

16. A method of obtaining a nucleic acid fragment encoding the amino acid sequence encoding a sodium channel agonist polypeptide comprising the steps of:

(a) probing a cDNA or genomic library with an isolated polynucleotide comprising a nucleotide sequence of at least one of 30 contiguous nucleotides of the isolated polynucleotide of Claim 1;

(b) identifying a DNA clone that hybridizes with the isolated polynucleotide;

(c) isolating the identified DNA clone; and

(d) sequencing the cDNA or genomic fragment that comprises the isolated DNA clone.

17. A recombinant baculovirus expression vector comprising an isolated polynucleotide of Claim 1.

18. An expression cassette comprising at least one nucleic acid of Claim 1 operably linked to a promoter.

19. A method for positive selection of a transformed cell comprising:

transforming a plant cell with the chimeric gene of claim 6 or the expression cassette of Claim 18; and

growing the transformed plant cell under conditions allowing expression of the polynucleotide in an amount sufficient to induce insect resistance to provide a positive selection means.

20. The method of Claim 19 wherein the plant cell is a dicot cell.

5

Figure 1

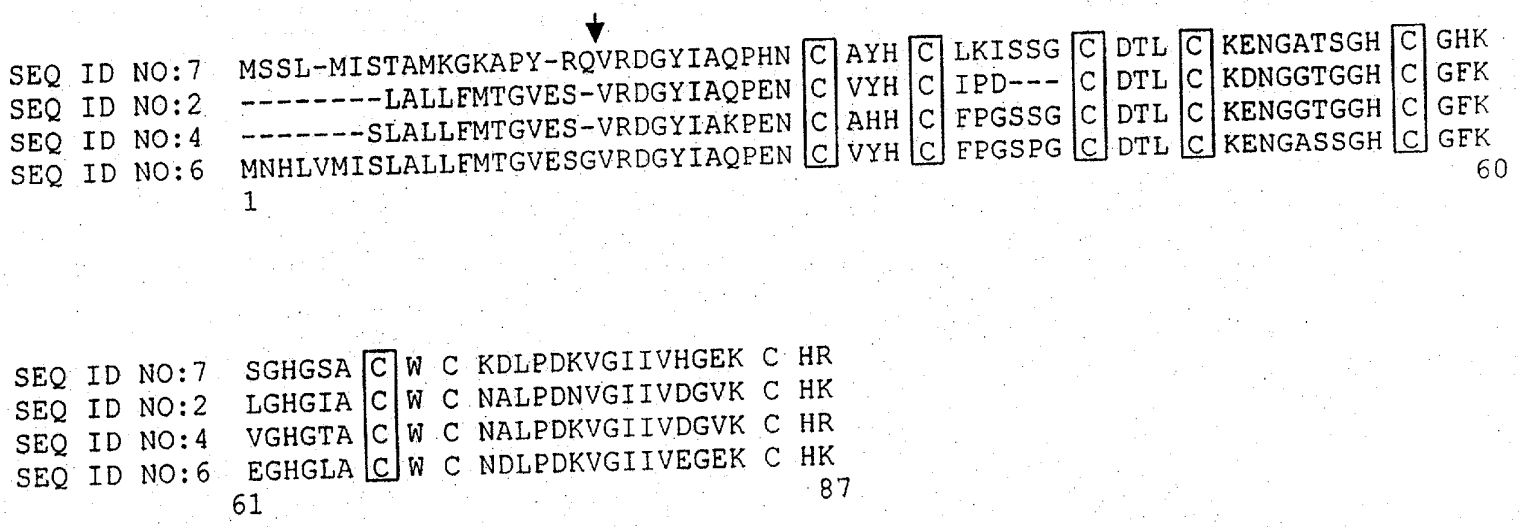
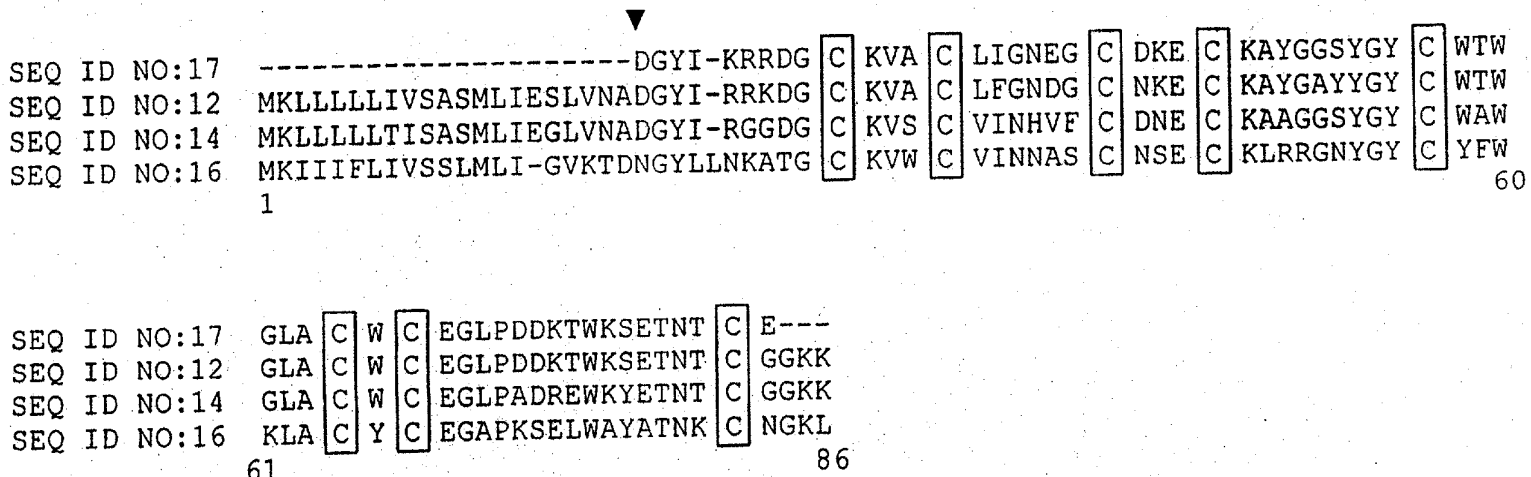

SEQ ID NO:7 MSSL-MISTAMKGKAPY-RQVRDGYIAQPHN C AYH C LKISSG C DTL C KENGATSGH C GHK
SEQ ID NO:2 --------LALLFMTGVES-VRDGYIAQPEN C VYH C IPD--- C DTL C KDNGGTGGH C GFK
SEQ ID NO:4 -------SLALLFMTGVES-VRDGYIAKPEN C AHH C FPGSSG C DTL C KENGGTGGH C GFK
SEQ ID NO:6 MNHLVMISLALLFMTGVESGVRDGYIAQPEN C VYH C FPGSPG C DTL C KENGASSGH C GFK
1
60
SEQ ID NO:7 SGHGSA C W C KDLPDKVGIIVHGEK C HR
SEQ ID NO:2 LGHGIA C W C NALPDNVGIIVDGVK C HK
SEQ ID NO:4 VGHGTA C W C NALPDKVGIIVDGVK C HR
SEQ ID NO:6 EGHGLA C W C NDLPDKVGIIVEGEK C HK
61
87

FIGURE 2

```
                                    ▼
SEQ ID NO:10  ----------GRDAYIAQPEN C VYE C AQNSY C NDL C TKNGATSGY C QWLGK
SEQ ID NO:09  MNYLVXISLALLLMTGVESGRDAYIAQNYN C VYH C ALNPY C NDL C TKNGAKSGY C QWFGS
                      1                                                      60

SEQ ID NO:10  YGNA C W C KDLPDNVPIRIPGK C HF
SEQ ID NO:09  SGNA C W C IDLPDNVPIKVPGK C HR
                 61                    84
```

FIGURE 3


SEQ ID NO:17 ----------DGYI-KRRDG C KVA C LIGNEG C DKE C KAYGGSYGY C WTW
SEQ ID NO:12 MKLLLLLIVSASMLIESLVNADGYI-RRKDG C KVA C LFGNDG C NKE C KAYGAYYGY C WTW
SEQ ID NO:14 MKLLLLLTISASMLIEGLVNADGYI-RGGDG C KVS C VINHVF C DNE C KAAGGSYGY C WAW
SEQ ID NO:16 MKIIIFLIVSSLMLI-GVKTDNGYLLNKATG C KVW C VINNAS C NSE C KLRRGNYGY C YFW
1
60
SEQ ID NO:17 GLA C W C EGLPDDKTWKSETNT C E---
SEQ ID NO:12 GLA C W C EGLPDDKTWKSETNT C GGKK
SEQ ID NO:14 GLA C W C EGLPADREWKYETNT C GGKK
SEQ ID NO:16 KLA C Y C EGAPKSELWAYATNK C NGKL
61
86

SEQUENCE LISTING

<110> E. I. du Pont de Nemours and Company

<120> SCORPION TOXINS

<130> BB1208

<140>
<141>

<150> 60/105,404
<151> 1998-10-23

<160> 17

<170> Microsoft Office 97

<210> 1
<211> 228
<212> DNA
<213> Leiurus quinquestriatus

<400> 1
gtttggcact tctcttcatg acaggtgtgg agagtgtacg tgatggttat attgcccagc 60
ccgaaaactg tgtctaccat tgcattccag attgcgacac gttatgtaag gataacggtg 120
gtacgggtgg ccattgcgga tttaaacttg gacacggaat tgcctgctgg tgcaatgcct 180
tgcccgataa tgtagggatt atagttgatg gagtaaaatg tcataaag 228

<210> 2
<211> 75
<212> PRT
<213> Leiurus quinquestriatus

<220>
<221> SIGNAL
<222> (1)..(11)

<400> 2
Leu Ala Leu Leu Phe Met Thr Gly Val Glu Ser Val Arg Asp Gly Tyr
1 5 10 15

Ile Ala Gln Pro Glu Asn Cys Val Tyr His Cys Ile Pro Asp Cys Asp
20 25 30

Thr Leu Cys Lys Asp Asn Gly Gly Thr Gly Gly His Cys Gly Phe Lys
35 40 45

Leu Gly His Gly Ile Ala Cys Trp Cys Asn Ala Leu Pro Asp Asn Val
50 55 60

Gly Ile Ile Val Asp Gly Val Lys Cys His Lys
65 70 75

<210> 3
<211> 238
<212> DNA
<213> Leiurus quinquestriatus

<400> 3
tagtttggca cttctcttca tgacaggngt ggagagtgta cgtgacggtt atattgccaa 60
gcccgaaaac tgtgcacacc attgctttcc agggtcctcc ggttgcgaca cattatgtaa 120
ggaaaacggt ggtacgggtg gccattgcgg atttaaagtt ggacatggaa ctgcctgctg 180
gtgcaatgcc ttgcccgata aagtagggat tatagtagat ggagtaaaat gccatcgc 238

<210> 4
<211> 79
<212> PRT
<213> Leiurus quinquestriatus

<220>
<221> SIGNAL
<222> (1)..(12)

<400> 4
Ser Leu Ala Leu Leu Phe Met Thr Gly Val Glu Ser Val Arg Asp Gly
1 5 10 15

Tyr Ile Ala Lys Pro Glu Asn Cys Ala His His Cys Phe Pro Gly Ser
20 25 30

Ser Gly Cys Asp Thr Leu Cys Lys Glu Asn Gly Gly Thr Gly Gly His
35 40 45

Cys Gly Phe Lys Val Gly His Gly Thr Ala Cys Trp Cys Asn Ala Leu
50 55 60

Pro Asp Lys Val Gly Ile Ile Val Asp Gly Val Lys Cys His Arg
65 70 75

<210> 5
<211> 258
<212> DNA
<213> Leiurus quinquestriatus

<400> 5
atgaatcatt tggtaatgat tagtttggca cttcttttca tgacaggtgt ggagagtggt 60
gtacgtgatg ggtatattgc ccagcccgaa aactgtgtct accattgctt tccagggtcc 120
cccggttgcg acacattatg taaagagaac ggtgcttcga gtggccattg cggatttaaa 180
gaaggacacg gacttgcctg ctggtgcaat gatctgcccg ataaagtagg gataatagta 240
gaaggagaaa aatgccat 258

<210> 6
<211> 87
<212> PRT
<213> Leiurus quinquestriatus

<220>
<221> SIGNAL
<222> (1)..(19)

<400> 6
Met Asn His Leu Val Met Ile Ser Leu Ala Leu Leu Phe Met Thr Gly
1 5 10 15

Val Glu Ser Gly Val Arg Asp Gly Tyr Ile Ala Gln Pro Glu Asn Cys
20 25 30

Val Tyr His Cys Phe Pro Gly Ser Pro Gly Cys Asp Thr Leu Cys Lys
35 40 45

Glu Asn Gly Ala Ser Ser Gly His Cys Gly Phe Lys Glu Gly His Gly
50 55 60

Leu Ala Cys Trp Cys Asn Asp Leu Pro Asp Lys Val Gly Ile Ile Val
65 70 75 80

Glu Gly Glu Lys Cys His Lys
85

<210> 7
<211> 85
<212> PRT
<213> Buthus occitanus

<400> 7
Met Ser Ser Leu Met Ile Ser Thr Ala Met Lys Gly Lys Ala Pro Tyr
1 5 10 15

Arg Gln Val Arg Asp Gly Tyr Ile Ala Gln Pro His Asn Cys Ala Tyr
20 25 30

His Cys Leu Lys Ile Ser Ser Gly Cys Asp Thr Leu Cys Lys Glu Asn
35 40 45

Gly Ala Thr Ser Gly His Cys Gly His Lys Ser Gly His Gly Ser Ala
50 55 60

Cys Trp Cys Lys Asp Leu Pro Asp Lys Val Gly Ile Ile Val His Gly
65 70 75 80

Glu Lys Cys His Arg
85

<210> 8
<211> 252
<212> DNA
<213> Leiurus quinquestriatus

<400> 8
atgaattatt tggtantgat tagtttggca cttctcctca tgacaggtgt ggagagtgga 60
cgtgatgctt atattgccca gaactataac tgtgtatatc attgtgcttt aaatccatat 120
tgcaacgatt tatgtaccaa gaacggtgct aagagtggct attgccaatg gttcggttca 180
agtggaaacg cctgctggtg catagatttg cccgataacg taccgattaa agtaccagga 240
aaatgccatc gc 252

<210> 9
<211> 84
<212> PRT
<213> Leiurus quinquestriatus

<220>
<221> SIGNAL
<222> (1)..(19)

<400> 9
Met Asn Tyr Leu Val Xaa Ile Ser Leu Ala Leu Leu Leu Met Thr Gly
1 5 10 15

Val Glu Ser Gly Arg Asp Ala Tyr Ile Ala Gln Asn Tyr Asn Cys Val
20 25 30

Tyr His Cys Ala Leu Asn Pro Tyr Cys Asn Asp Leu Cys Thr Lys Asn
35 40 45

Gly Ala Lys Ser Gly Tyr Cys Gln Trp Phe Gly Ser Ser Gly Asn Ala
50 55 60

Cys Trp Cys Ile Asp Leu Pro Asp Asn Val Pro Ile Lys Val Pro Gly
65 70 75 80

Lys Cys His Arg

<210> 10
<211> 65

<212> PRT
<213> Buthus occitanus tunetanus

<400> 10
Gly Arg Asp Ala Tyr Ile Ala Gln Pro Glu Asn Cys Val Tyr Glu Cys
1 5 10 15

Ala Gln Asn Ser Tyr Cys Asn Asp Leu Cys Thr Lys Asn Gly Ala Thr
20 25 30

Ser Gly Tyr Cys Gln Trp Leu Gly Lys Tyr Gly Asn Ala Cys Trp Cys
35 40 45

Lys Asp Leu Pro Asp Asn Val Pro Ile Arg Ile Pro Gly Lys Cys His
50 55 60

Phe
65

<210> 11
<211> 256
<212> DNA
<213> Leiurus quinquestriatus

<400> 11
atgaaactct tacttttact cattgtctct gcttcaatgc tgattgaaag cttagttaat 60
gctgacggat atataagaag aaaagacgga tgcaaggttg catgcctgtt cggaaatgac 120
ggctgcaata aagaatgcaa agcttatggt gcctattatg gatattgttg gacctgggga 180
cttgcctgct ggtgcgaagg tcttccggat gacaagacat ggaagagtga aacaaacaca 240
tgcggtggca aaaagt 256

<210> 12
<211> 85
<212> PRT
<213> Leiurus quinquestriatus

<220>
<221> SIGNAL
<222> (1)..(21)

<400> 12
Met Lys Ile Ile Ile Phe Leu Ile Val Ser Ser Leu Met Leu Ile Gly
1 5 10 15

Val Lys Thr Asp Asn Gly Tyr Leu Leu Asn Lys Ala Thr Gly Cys Lys
20 25 30

Val Trp Cys Val Ile Asn Asn Ala Ser Cys Asn Ser Glu Cys Lys Leu
35 40 45

Arg Arg Gly Asn Tyr Gly Tyr Cys Tyr Phe Trp Lys Leu Ala Cys Tyr
50 55 60

Cys Glu Gly Ala Pro Lys Ser Glu Leu Trp Ala Tyr Ala Thr Asn Lys
65 70 75 80

Cys Asn Gly Lys Leu
85

<210> 13
<211> 255
<212> DNA
<213> Leiurus quinquestriatus

<400> 13
atgaaactgt tacttctgct aactatctca gcttcaatgc tgattgaagg cttagttaat 60
gctgacggat atataagagg aggcgacgga tgcaaggttt catgcgtgat aaatcatgtg 120
ttttgtgata atgaatgcaa agctgctggt ggctcttatg gatattgttg ggcctgggga 180
cttgcctgct ggtgcgaagg tcttccagct gacagggaat ggaagtatga aaccaataca 240
tgcggtggca aaaag 255

<210> 14
<211> 85
<212> PRT
<213> Leiurus quinquestriatus

<220>
<221> SIGNAL
<222> (1)..(21)

<400> 14
Met Lys Leu Leu Leu Leu Leu Thr Ile Ser Ala Ser Met Leu Ile Glu
1 5 10 15

Gly Leu Val Asn Ala Asp Gly Tyr Ile Arg Gly Gly Asp Gly Cys Lys
20 25 30

Val Ser Cys Val Ile Asn His Val Phe Cys Asp Asn Glu Cys Lys Ala
35 40 45

Ala Gly Gly Ser Tyr Gly Tyr Cys Trp Ala Trp Gly Leu Ala Cys Trp
50 55 60

Cys Glu Gly Leu Pro Ala Asp Arg Glu Trp Lys Tyr Glu Thr Asn Thr
65 70 75 80

Cys Gly Gly Lys Lys
85

<210> 15
<211> 255
<212> DNA
<213> Leiurus quinquestriatus

<400> 15
atgaaaataa taatttttct aattgtgtca tcattaatgc tgataggagt gaagaccgat 60
aatggttact tgcttaacaa agccaccggt tgcaaggtct ggtgtgttat taataatgca 120
tcttgtaata gtgagtgtaa actaagacgt ggaaattatg gctactgcta tttctggaaa 180
ttggcctgtt attgcgaagg agctccaaaa tcagaacttt gggcttacgc aaccaataaa 240
tgcaatggga aatta 255

<210> 16
<211> 85
<212> PRT
<213> Leiurus quinquestriatus

<220>
<221> SIGNAL
<222> (1)..(19)

<400> 16
Met Lys Leu Leu Leu Leu Leu Ile Val Ser Ala Ser Met Leu Ile Glu
1 5 10 15

Ser Leu Val Asn Ala Asp Gly Tyr Ile Arg Arg Lys Asp Gly Cys Lys
20 25 30

Val Ala Cys Leu Phe Gly Asn Asp Gly Cys Asn Lys Glu Cys Lys Ala
35 40 45

Tyr Gly Ala Tyr Tyr Gly Tyr Cys Trp Thr Trp Gly Leu Ala Cys Trp
50 55 60

Cys Glu Gly Leu Pro Asp Asp Lys Thr Trp Lys Ser Glu Thr Asn Thr
65 70 75 80

Cys Gly Gly Lys Lys
85

<210> 17
<211> 61
<212> PRT
<213> Leiurus quinquestriatus

<400> 17
Asp Gly Tyr Ile Lys Arg Arg Asp Gly Cys Lys Val Ala Cys Leu Ile
1 5 10 15

Gly Asn Glu Gly Cys Asp Lys Glu Cys Lys Ala Tyr Gly Gly Ser Tyr
20 25 30

Gly Tyr Cys Trp Thr Trp Gly Leu Ala Cys Trp Cys Glu Gly Leu Pro
35 40 45

Asp Asp Lys Thr Trp Lys Ser Glu Thr Asn Thr Cys Glu
50 55 60

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
BB1208 PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/24922	22/10/1999	23/10/1998
International Patent Classification (IPC) or national classification and IPC C07K14/00		
Applicant E. I. DU PONT DE NEMOURS AND COMPANY et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
28/04/2000	04.01.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Donath, C Telephone No. +49 89 2399 8710

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US99/24922

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-25 as originally filed

Claims, No.:

1-20 as originally filed

Drawings, sheets:

1/3-3/3 as originally filed

Sequence listing part of the description, pages:

1-6, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:
see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☐ all parts.

☒ the parts relating to claims Nos. 1-20 (partially) referring to SEQ ID NOs: 1 and 2.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N) Yes: Claims 1-20

	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-20
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-20
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Ad section IV.:

This International Preliminary Examining Authority agrees with the International search Authority in that this International application contains multiple (groups of) inventions, which are:

1. Claims: 1-20 (partially)
Claims 1 - 20 (partially) refer to a scorpion alpha toxin XIV polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:2 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

2. Claims: 1-20 (partially)
Claims 1 - 20 (partially) refer to a scorpion alpha toxin XIV polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:4 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

3. Claims: 1-20 (partially)
Claims 1 - 20 (partially) refer to a scorpion alpha toxin XIV polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:6 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

4. Claims: 1-20 (partially)
Claims 1 - 20 (partially) refer to a scorpion neurotoxin I polypeptide having at least 95%

identity as compared to the polypeptide of SEQ ID NO:9 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

5. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion depressant toxin LqhIT2 polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:12 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

6. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion depressant toxin LqhIT2 polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:14 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

7. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion depressant toxin LqhIT2 polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:16 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

The general inventive concept underlying the 3 groups of scorpion toxin polypeptides -

the scorpion alpha toxin XIV polypeptides, the scorpion neurotoxin I polypeptide, and the scorpion depressant toxin LqhIT2 polypeptides - of the present application can be seen as scorpion toxin polypeptides acting as sodium channel agonists. However, this general inventive concept is not novel having regard to the prior art as illustrated e.g. by document WO 96/36712 or Eur.J.Biochem. 238,653-660 (1996), which both disclose scorpion toxin polypeptides capable of acting as sodium channel agonists.
Moreover, scorpion toxin polypeptides being a member of one of the three above mentioned groups of scorpion toxin polypeptides are already known from the prior art; e.g. the polypeptides described in Eur.J.Biochem. 238,653-660 (1996) belong to the group of scorpion alpha toxin XIV polypeptides, some of the polypeptides listed in WO 96/36221 belong to the group of scorpion neurotoxin I polypeptides, and the polypeptides described in WO 96/36781 or those disclosed in Eur.J.Biochem. 254,44-49 (1998) belong to the group of scorpion depressant toxin LqhIT2 polypeptides.
Therefore, a single general inventive concept is not acceptable, making necessary to reconsider the technical relationship or interaction between the different inventions mentioned.
This leads to their regrouping under different subjects as listed above, each subject is falling under its own inventive concept, being a solution to the problem in a way which differs from the state of the art.
As the applicant has not had an International search report drawn up on the other inventions, the International application can only be prosecuted on the basis of the invention in respect of which a search has already been carried out, in other words the invention first mentioned above.

Ad section V.:

1. The present International application refers to a scorpion alpha toxin XIV polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:2 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

In view of the documents cited in the International Search Report the subject-matter of claims 1 - 20 of the present International application has to be regarded as being new (Article 33(2) PCT).

2. The closest prior art to evaluate the inventiveness of the subject-matter of the present International application is document Eur.J.Biochem. 238,653-660 (1996). This document discloses the cloning of a DNA sequence encoding an alpha toxin XIV from the scorpion *Buthus occitanus tunetanus*. The toxin, called Bot XIV, displays no toxicity towards mammals but is active towards insects as shown by its paralytic activity against cockroaches.
In the prior art no indication has been given for a further scorpion alpha toxin XIV polypeptide, specially for an alpha toxin XIV consisting of a polypeptide of SEQ ID NO:2 and for a nucleotide sequence encoding the same.
Thus, an inventive step has to be acknowledged for claims 1 - 20 of the present International application (Article 33(3) PCT).

Ad section VIII.:

1. Claims 12, 15 and 16 lack clarity due to the expression "...polynucleotide comprising a nucleotide sequence of **at least one of 30 contiguous** nucleotides...". This term is not suitable to clearly define the scope of the claims. It is absolutely ambiguous whether the polynucleotide has to comprise at least 30 contiguous nucleotides derived from the isolated polynucleotide of claim 1 or whether even **less** than 30 contiguous nucleotides derived from the isolated polynucleotide of claim 1 may be thought to be sufficient.
The applicant is already informed that novelty for the subject-matter of these claims can only be acknowledged when the claims are interpreted as to refer to a polynucleotide comprising at least 30 contiguous nucleotides derived from the isolated polynucleotide of claim 1.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
BB1208 PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 99/24922	22/10/1999	23/10/1998
Applicant		
E. I. DU PONT DE NEMOURS AND COMPANY et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 6 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [X] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [X] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

   [X] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

national application No.
PCT/US 99/24922

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1 - 20 (partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion alpha toxin XIV polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:2 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

2. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion alpha toxin XIV polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:4 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

3. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion alpha toxin XIV polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:6 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

4. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion neurotoxin I polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:9 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

5. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion depressant toxin LqhIT2 polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:12 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

6. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion depressant toxin LqhIT2 polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:14 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

7. Claims: 1-20 (partially)

Claims 1 - 20 (partially) refer to a scorpion depressant toxin LqhIT2 polypeptide having at least 95% identity as compared to the polypeptide of SEQ ID NO:16 and which polypeptide is acting as a scorpion sodium channel agonist. Furthermore, the claims concern a chimeric gene encoding all or a portion of said scorpion sodium channel agonist, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the scorpion sodium channel agonist in a transformed host cell.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C12N15/86 C12N7/01 C12N5/10 C07K14/435
A01N63/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, STRAND, MEDLINE, EMBASE, BIOSIS, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 36712 A (E.I. DU PONT DE NEMOURS AND COMPANY) 21 November 1996 (1996-11-21) page 3, line 10 - line 35; examples 1-4 ---	1-20
A	WO 96 36221 A (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 21 November 1996 (1996-11-21) page 6, line 28 -page 19, line 2 ---	1-20
A	SAUTIÈRE, P. ET AL.: "New toxins acting on sodium channels from the scorpion Leiurus Quinquestriatus Hebraeus suggest a clue to mammalian vs insect selectivity" TOXICON, vol. 36, no. 8, August 1998 (1998-08), pages 1141-1154, XP000916899 page 1145 -page 1152 'Results and Discussion' ---	1-20

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 July 2000	23. 11. 00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Donath, C

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MOSKOWITZ, H. ET AL.: "A depressant insect-selective toxin analog from the venom of the scorpion Leiurus quinquestriatus hebraeus, purification and structure/function characterization" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 254, no. 1, 15 May 1998 (1998-05-15), pages 44-49, XP000919258 page 45 -page 49 'Results' and 'Discussion' ---	1-20
A	BOUHAOUALA-ZAHAR, B. ET AL.: "A recombinant insect-specific alpha-toxin of Buthus occitanus tunetanus scorpion confers protection against homologous mammal toxins" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 238, no. 3, 15 June 1996 (1996-06-15), pages 653-660, XP000919257 page 655 -page 659 'Results' and 'Discussion' -----	1-20

Infor. n on patent family members

Int ional Application No
PC1/US 99/24922

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9636712 A	21-11-1996	AU 723612 B	31-08-2000
		AU 5865196 A	29-11-1996
		BR 9608741 A	06-07-1999
		CN 1184504 A	10-06-1998
		EP 0826047 A	04-03-1998
		JP 10509596 T	22-09-1998
		NZ 308772 A	29-04-1999
		US 6096304 A	01-08-2000
WO 9636221 A	21-11-1996	US 5756340 A	26-05-1998
		AU 710774 B	30-09-1999
		AU 5788796 A	29-11-1996
		BR 9608474 A	13-10-1999
		CN 1185718 A	24-06-1998
		EP 0838999 A	06-05-1998
		JP 11501521 T	09-02-1999
		NZ 308294 A	27-05-1998

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/12, 15/86, A01N 63/02 — A1

(11) International Publication Number: WO 96/36712

(43) International Publication Date: 21 November 1996 (21.11.96)

(21) International Application Number: PCT/US96/06988

(22) International Filing Date: 16 May 1996 (16.05.96)

(30) Priority Data:
08/443,294 17 May 1995 (17.05.95) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/443,294 (CIP)
Filed on 17 May 1995 (17.05.95)

(71) Applicant *(for all designated States except US)*: E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: MCCUTCHEN, Billy, Fred [US/US]; 105 Monet Circle, Wilmington, DE 19808-1123 (US).

(74) Agent: FLOYD, Linda, A.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).

(81) Designated States: AL, AM, AU, AZ, BB, BG, BR, BY, CA, CN, CZ, EE, GE, HU, IS, JP, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TR, TT, UA, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.
With an indication in relation to a deposited microorganism furnished under Rule 13bis separately from the description.
Date of receipt by the International Bureau:
17 June 1996 (17.06.96)

(54) Title: RECOMBINANT BACULOVIRUS INSECTICIDES

LqhIT	NPV	GACGGCTACA	TCAAACGCCG	CGACGGCTGC	AAAGTGGCCT	40
LqhIT	cDNA	GACGGATATA	TAAAAAGACG	AGACGGATGC	AAGGTTGCAT	40
LqhIT	NPV	GCCTTATCGG	CAACGAGGGC	TGCGACAAAG	AGTGCAAGGC	80
LqhIT	cDNA	GCCTGATCGG	AAATGAGGGC	TGCGATAAAG	AATGCAAAGC	80
LqhIT	NPV	CTACGGCGGC	AGCTACGGCT	ACTGCTGGAC	CTGGGGCCTC	120
LqhIT	cDNA	TTATGGTGGC	TCTTATGGAT	ATTGTTGGAC	CTGGGGACTT	120
LqhIT	NPV	GCATGCTGGT	GCGAGGGCCT	CCCCGACGAC	AAAACCTGGA	160
LqhIT	cDNA	GCCTGCTGGT	GCGAAGGTCT	TCCGGATGAC	AAGACATGGA	160
LqhIT	NPV	AAAGCGAAAC	CAACACCTGC	GGCTAA		186
LqhIT	cDNA	AGAGTGAAAC	AAACACATGC	GGTTAA		186

(57) Abstract

This invention pertains to recombinant baculoviruses that have been engineered to afford optimal expression of genes encoding insect-selective neurotoxins. More specifically, this invention pertains to an isolated nucleic acid sequence encoding the scorpion toxin LqhIT2, derived from *Leiurus quinquestriatus hebraeus*, wherein said sequence has been optimized for gene expression in nuclear polyhedrosis virus-infected cells. This invention also pertains to chimeric genes comprising a codon-optimized LqhIT2 nucleotide sequence, insecticidal compositions comprising recombinant baculoviruses expressin a codon-optimized, insect-selective neurotoxin (e.g., the LqhIT2 toxin gene), and methods for controlling insects in both agronomic and non-agronomic environments comprising application of insect baculoviruses containing the codon-optimized nucleic acid sequence encoding an insect-selective neurotoxin such as the LqhIT2 toxin.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

TITLE

RECOMBINANT BACULOVIRUS INSECTICIDES

BACKGROUND OF THE INVENTION

Chemical insecticides are an integral component of modern agriculture, and have afforded an effective means for reducing crop damage by controlling insect pests. However, chemical agents are under continuous scrutiny due to the potential for environmental contamination, selection of resistant populations of agronomic pests, and toxicity to non-target organisms such as beneficial insects, aquatic organisms, animals and man. As a result, alternative strategies for insect control are being sought that are effective and yet benign to non-target populations and the environment. One of these strategies comprises the use of microorganisms that are naturally occurring pathogens of target insect populations. However, many candidate entomopathogens that would be promising insect control agents lack the properties of classical chemical insecticides that farmers and others in agribusiness have grown accustomed to. For instance, insect-specific viruses from the family Baculoviridae possess several favorable attributes, including host-specificity and inert environmental properties, but lack the ability to rapidly control a target population before significant crop damage takes place. Fortunately, modern molecular biology provides the tools necessary to favorably modify many of these properties in order to satisfy the needs of modern agriculture.

Baculoviruses are viruses pathogenic to invertebrates, and are characterized by possession of a double-stranded, circular DNA genome ranging in size from 80 to 200 kilobases. Baculoviruses are divided into three subfamilies, including non-occluded baculoviruses (NOVs), granulosis viruses (GVs) and nuclear polyhedrosis viruses (NPVs). Examples of NOVs are *Orcytes rhinoceros* NOV and *Helicoverpa zea* NOV. Examples of GVs include *Plutella xylostella* GV, *Cydia pomonella* GV, *Pieris brassicae* GV, and *Trichoplusia ni* GV. Examples of NPVs include *Autographa californica* NPV, *Spodoptera exigua* NPV, *Heliothis armigera* NPV, *Helicoverpa zea* NPV, *Spodoptera frugiperda* NPV, *Trichoplusia* NPV, *Mamestra brassicae* NPV, *Lymantria dispar* NPV, *Spodoptera litturalis* NPV, *Syngrapha facifera* NPV, *Choristoneura fumiferana* NPV, *Anticarsia gemmatalis* NPV, and *Heliothis virescens* NPV.

Although certain GVs and NOVs have been carefully studied, NPVs are the most thoroughly characterized of the baculovirus subfamilies. The infection cycle of NPVs involves two types of virions. Following infection of insect cells, budded virions (BVs or extra cellular virus, ECV) are produced upon movement of nucleocapsids to the plasma membrane. These virions shed their nuclear-derived coat in the cytoplasm and bud through the cytoplasmic membrane into the hemocoel of the insect host. This process leads to systemic infection of the host insect. Later in the infection process, virions

become occluded (occluded virions) within a protein matrix consisting substantially of the polyhedrin protein, thus forming polyhedrin inclusion bodies (PIBs or occlusion bodies, OBs). These inclusion bodies are the orally infectious form of the virus, and provide for horizontal transmission of the virus between insect hosts (1,2). Uninfected larvae feed on virus-contaminated substrates and ingest PIBs. The proteinaceous matrix is solubilized by the action of the basic pH of the insect midgut found in many lepidopterous larvae. The liberated virion nucleocapsids, containing the viral DNA genome, attach to and infect the epithelial cells of the larval midgut. Typically, the infected insect will continue to develop and consume plant material while the virus exponentially propagates within the host. Eventually, often after several weeks or longer have passed, the infected larvae will become fully involved and expire.

An attractive attribute of baculoviruses is their narrow host specificity. These viruses infect only arthropods, and possess relatively narrow host ranges even within a particular insect order. Host specificity has been examined by electron microscopy, DNA hybridization and recombinant DNA technology (3-5). These studies indicate that the narrow host range is due, at least in part, to the inability of baculoviruses to transfer viral DNA into the mammalian cell nucleus.

Due in part to the availability of efficient cell culture systems and facile cloning vectors, NPVs have been utilized as eukaryotic expression vectors for synthesis of desirable heterologous proteins (6,7). One virus in particular, *Autographa californica* NPV (AcNPV), is the accepted model virus utilized for introduction and expression of heterologous genes in baculovirus expression systems. Although this virus is routinely used as an important *in vitro* means of providing for high yields of recombinant proteins in a eukaryotic expression system, thus affording appropriate post-translational modification of expressed proteins, AcNPV is capable of infecting many families of Lepidopteran insects that are important economic pests.

In spite of the potential practical advantages of baculovirus-based pest control agents, a variety of disadvantages have curtailed their use in modern agriculture. The most significant barrier to more widespread use of these viruses in row-crop agriculture is the significant time delay between their application and effective control of crop damage caused by the host insects. Unlike the rapid effects observed upon application of classical chemical insecticides, effective wild-type baculovirus-mediated insect control occurs only after *in vivo* populations of virus have reached levels high enough to compromise host activity. However, through the use of recombinant DNA technology, NPVs have been genetically engineered to increase their rate of insect killing by either the introduction of genes directing the expression of insecticidal proteins, or deletion of genes from the viral genome (8-10). The most effective recombinant NPVs have been

engineered to express insect-selective neurotoxins (11-18). These recombinant viruses kill their hosts in 20-30% less time than wild-type NPVs.

There has now been constructed recombinant NPVs that have significantly greater potency than previously constructed recombinant NPVs. These recombinant NPVs have been engineered to express a heterologous gene encoding the insect-selective toxin LqhIT2 of the scorpion *Leiurus quinquestriatus hebraeus* (19,20). Based on present studies, the recombinant NPVs carrying this synthetic gene provide for a significant increase in the insecticidal properties of the virus.

SUMMARY OF THE INVENTION

This invention pertains to recombinant baculoviruses that have been engineered to afford optimal expression of genes encoding insect-selective neurotoxins. More specifically, this invention pertains to an isolated nucleic acid sequence encoding the scorpion toxin LqhIT2, derived from *Leiurus quinquestriatus hebraeus*, wherein said sequence has been optimized for gene expression in nuclear polyhedrosis virus-infected cells. This invention also pertains to chimeric genes comprising a codon-optimized LqhIT2 nucleotide sequence, insecticidal compositions comprising recombinant baculoviruses expressing a codon-optimized, insect-selective neurotoxin such as the LqhIT2 toxin gene, and methods for controlling insects in both agronomic and non-agronomic environments comprising application of insect baculoviruses containing a codon-optimized, insect-selective neurotoxin such as the LqhIT2 toxin gene.

The insect baculoviruses of the instant invention are selected from the group nuclear polyhedrosis viruses, singly or multiply occluded nuclear polyhedrosis viruses and granulosis viruses. Preferred baculoviruses are selected from the group multinucleocapsid nuclear polyhedrosis viruses. Specifically preferred is *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV).

The instant invention comprises a synthetic gene encoding the LqhIT2 protein wherein codon selection is biased towards codons favored by nuclear polyhedrosis viruses and cells that support their replication, as determined by observation of codon utilization in genes encoding a well characterized nuclear polyhedrosis virus protein, polyhedrin, and several lepidopteran proteins. The resulting genetic constructs afford efficient expression of LqhIT2 in cells infected with recombinant baculoviruses harboring the synthetic LqhIT2 gene. Application of these recombinant viruses to *Heliothis virescens* results in rapid paralysis of treated larvae. Moreover, viruses containing the codon-biased gene kill their insect hosts more rapidly than viruses containing a complimentary DNA (cDNA) copy of the LqhIT2 gene.

BRIEF DESCRIPTION OF THE DRAWINGS, BIOLOGICAL DEPOSITS AND SEQUENCE LISTING

Figure 1. Alignment of the cDNA sequence of the LqhIT2 gene from *Leiurus quinquestriatus hebraeus* (LqhIT cDNA) and the codon-biased, synthetic structural gene encoding LqhIT2 (LqhIT NPV). Bold letters indicate silent nucleotide changes to the cDNA sequence that were introduced in order to facilitate gene expression.

Figure 2. Sequence of the synthetic oligonucleotides used to construct the codon-biased form of the LqhIT2 gene. Oligonucleotide Lq1 encodes the Bombyxin signal peptide. Oligonucleotides Lq1 and Lq10 were used as primers for PCR amplification of the synthetic gene.

Figure 3. Diagramatic representation of the strategy employed for preparation of the codon-biased form of the LqhIT2 gene. Oligonucleotides Lq1 and Lq10 (marked with an "X") served as amplification primers for PCR reactions. Unique restriction enzyme cleavage sites are indicated.

Figure 4. Nucleotide and corresponding amino acid sequences of the codon-biased LqhIT2 gene. Lower case letters in the nucleotide sequence (nucleotides 1-57, encoding amino acids 1-19) indicate nucleotides encoding the Bombyxin signal peptide.

Figure 5. Plasmid maps of pTZ18R.LqhIT2 (intermediate cloning vector comprising the synthetic, codon-biased LqhIT2 gene), pAcUW21 (the baculovirus transfer vector), and derivation of the plasmid pAcUW21.LqhIT2, a baculovirus transfer vector comprising the synthetic, codon-biased LqhIT2 gene.

Figure 6. Graphic representation of the time to mortality (Lethal Time) of 3rd instar larvae of *H. virescens* treated with AcLqhIT2 and control viruses.

Figure 7. Graphic representation of inhibition of plant destruction (i.e., plant protection) by larvae of *H. virescens* treated with wild-type and recombinant baculoviruses. Data are reported as percent of leaf material remaining relative to control (uninfested) plants.

The present invention further comprises recombinant baculoviruses that have been deposited under the terms of the Budapest Treaty at American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852, and bear the following accession numbers:

Recombinant Baculovirus	Accession Number	Date of Deposit
AcLqhIT2	ATCC VR-2501	May 2, 1995
CG201-3-1	ATCC VR-2502	May 2, 1995

Applicant has provided 13 sequence listings in conformity with "Rules for the Standard Representation of Nucleotide and Amino Acid sequences in Patent Applications" (Annexes I and II to the Decision of the President of the EPO, published in

Supplement No. 2 to OJ EPO, 12/1992) and with 37 C.F.R. 1.821-1.825 and Appendices A and B ("Requirements for Application Disclosures Containing Nucleotides and/or Amino Acid Sequences").

DETAILED DESCRIPTION OF THE INVENTION

In the context of the present disclosure, a number of terms and abbreviations shall be used. "NPV" stands for Nuclear Polyhedrosis Virus. "PIBs" are polyhedrin inclusion bodies. "AcNPV" stands for the wild-type *Autographa californica* Nuclear Polyhedrosis Virus. "LqhIT2" represents the insect-selective neurotoxin derived from *Leiurus quinquestriatus hebraeus*. "AaIT" represents the insect-selective neurotoxin derived from *Androctonus australis*. "AcLqhIT2" is a short-hand form representing AcNPV that has been genetically modified to contain the gene encoding LqhIT2 under the transcriptional control of the baculovirus late P10 promoter. "AcAaIT" is a short-hand form representing AcNPV that has been genetically modified to harbor the gene encoding AaIT.

"Expression" refers to the transcription and translation of a structural gene to yield the encoded protein. As will be appreciated by those skilled in the art, structural gene expression levels are affected by the regulatory sequences (promoter, polyadenylation sites, enhancers, etc.) employed and by the host cell in which the structural gene is expressed.

As used herein, suitable "regulatory sequences" refer to nucleotide sequences located upstream (5'), within, and/or downstream (3') to a structural gene, which control the transcription and/or expression of the coding sequences, potentially in conjunction with the protein biosynthetic apparatus of the cell. These regulatory sequences include promoters, enhancer elements, transcription termination sequences, and polyadenylation sequences.

"Promoter" refers to the nucleotide sequences at the 5' end of a structural gene which direct the initiation of transcription. Promoter sequences are necessary, but not always sufficient, to drive the expression of a downstream gene. Usually promoters drive transcription preferentially in the downstream direction, although promotional activity can be demonstrated (at a reduced level of expression) when the gene is placed upstream of the promoter. The level of transcription is regulated by promoter sequences. Thus, in the construction of heterologous promoter/structural gene combinations, the structural gene is placed under the regulatory control of a promoter such that the expression of the gene is controlled by promoter sequences. The promoter is positioned preferentially upstream to the structural gene and at a distance from the transcription start site that approximates the distance between the promoter and the gene it controls in its natural setting. As is known in the art, some variation in this distance can be tolerated without loss of promoter function.

The "3' non-coding sequences" refers to the portion of the DNA sequence of a gene that contains a polyadenylation signal and any other regulatory signal capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor.

As used herein, "gene" refers to the entire DNA sequence portion involved in the synthesis of a protein. A gene embodies the structural or coding portion of DNA which begins at the 5' end from the translational start codon (usually ATG) and extends to the stop (TAG, TGA or TAA) codon at the 3' end. It also contains a promoter region, usually located 5' or upstream to the structural gene, which initiates and regulates the expression of a structural gene. Also included in a gene are the 3' non-coding sequences. "Chimeric gene" refers to a gene comprising heterogeneous regulatory and coding sequences. A "heterologous gene" refers to a gene not normally found in the host organism but that is introduced by gene transfer.

"Structural gene" is that portion of a gene comprising a DNA segment encoding a protein, polypeptide or a portion thereof, and excluding the 5' and 3' sequences involved in regulatory control of gene expression. The structural gene may be one which is normally found in the cell or one which is not normally found in the cellular location wherein it is introduced, in which case it is termed a heterologous gene. A heterologous gene may be derived in whole or in part from any source known to the art, including a bacterial genome or episome, eukaryotic, nuclear or plasmid DNA, cDNA, viral DNA or chemically synthesized DNA. A structural gene may contain one or more modifications in either the coding or the untranslated regions which could affect the biological activity or the chemical structure of the expression product, the rate of expression or the manner of expression control. Such modifications include, but are not limited to, mutations, insertions, deletions and substitutions of one or more nucleotides. The structural gene may constitute an uninterrupted coding sequence or it may include one or more introns, bounded by the appropriate splice junctions. The structural gene may be a composite of segments derived from a plurality of sources, naturally occurring or synthetic. The structural gene may also encode a fusion protein.

"Synthetic gene" refers to a DNA sequence of a structural gene that is chemically synthesized in its entirety or for the greater part of the coding region. As exemplified herein, oligonucleotide building blocks are synthesized using procedures known to those skilled in the art and are ligated and annealed to form gene segments which are then enzymatically assembled to construct the entire gene. As is recognized by those skilled in the art, functionally and structurally equivalent genes to the synthetic genes described herein may be prepared by site-specific mutagenesis or other related methods used in the art.

The term "operably linked" refers to nucleic acid sequences on a single nucleic acid molecule which are associated so that the function of one is affected by the other. For example, a promoter is operably linked with a structure gene when it is capable of affecting the expression of that structural gene (i.e., that the structural gene is under the transcriptional control of the promoter).

"Transfection" refers to stably introducing a DNA segment carrying a functional gene into an organism that did not previously contain that gene. "Co-transfection" refers to simultaneous introduction of more than one DNA segment into an organism.

"Codon-bias" refers to the preference exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. When synthesizing a gene for improved expression in a host cell it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell. "Non-codon-biases" refers to unbiased, natural, native or wild-type.

"Chemically synthesized", as related to a sequence of DNA, means that the component nucleotides were assembled *in vitro*. Manual chemical synthesis of DNA may be accomplished using well established procedures (21), or automated chemical synthesis can be performed using one of a number of commercially available machines.

The present invention concerns construction and utilization of recombinant baculovirus insecticides, engineered to express the insect-selective neurotoxins. Disclosed is a synthetic gene encoding the authentic LqhIT2 toxin wherein codon usage has been biased towards codons frequently employed in highly expressed baculovirus genes in lepidopteran insects. Although the DNA sequence of this synthetic gene differs from the native LqhIT2 coding sequence, the encoded amino acid sequence is identical to the native toxin amino acid sequence. A chimeric gene comprising a baculovirus promoter, a nucleotide fragment encoding a signal peptide facilitating secretion of the expressed toxin, and the synthetic nucleic acid fragment encoding the toxin, was inserted into the genome of a nuclear polyhedrosis virus. Expression of this chimeric gene resulted in efficient toxin expression and enhancement of the insecticidal properties of the recombinant virus relative to non-recombinant baculoviruses. This improved activity is manifested by more rapid control of the target insect population and results in a significant reduction in feeding damage to crops caused by these insects. Surprisingly, the instant recombinant baculoviruses also displayed more rapid insect control than recombinant baculoviruses engineered to express another insect-selective toxin, AaIT.

Baculovirus insecticides have great potential to provide an environmentally benign method for agricultural insect pest control. However, improvements to efficacy are required in order to make these agents competitive with current chemical pest control agents. One approach for making such improvements is through genetic alteration of the virus. For instance, it may be possible to modify the viral genome in order to improve

the host range of the virus, to increase the environmental stability and persistence of the virus or to improve the infectivity and transmission of the virus. In addition, improving the rate at which the virus acts to compromise the infected insect would significantly enhance the attractiveness of baculovirus insecticides as adjuncts or replacements for chemical pest control agents. One method for increasing the speed with which the virus affects its insect host is to introduce foreign genes that encode proteins that are toxic to the insect wherein death or incapacitation of the insect is no longer dependent solely on the course of the viral infection, but instead is aided by the accumulation of toxic levels of the foreign protein.

Many arthropods produce a mixture of substances referred to as venom. These substances are synthesized in specialized glandular tissues, which, when directed by a stinging or piercing apparatus, are capable of paralyzing the arthropod's prey. Slow moving or stationary arthropods have adapted a strategy to instantaneously paralyze their prey by utilizing neurotoxic components of the venom at very low concentrations. These components or neurotoxins interfere with the function of insect nervous tissues through efficient competition for certain receptor sites. Many of these neurotoxins are polypeptides; these have been divided into different classes based on their host specificity and mode of action (22). For example, neurotoxic peptides isolated from numerous species of scorpions have been divided into classes that affect arthropods and classes that affect mammals.

Several of the arthropod-specific toxins have been identified as insect-selective peptides. For example, the *Buthinae* scorpions express two types of insect-selective neurotoxins that contrast in their biological effects on target insects. In the blowfly, those classified as excitatory toxins cause immediate, fast and reversible contractive paralysis caused by the induction and repetitive firing of the terminal branches of the motor neurons (23-25). These toxins are single-chained polypeptides of approximately 70 amino acids, and are cross-linked by four disulfide bridges. The excitatory effect is attributed to increased sodium conductance, and a voltage-dependent slowing of the channel's closure, resulting in negative discharges in effected neurons. AaIT, a toxin produced from the venom of the scorpion *Androctonus australis* (26), was the first insect toxin isolated from these organisms that exhibited this excitatory action.

A second class of insect-selective neurotoxins are the depressant toxins, including BjIT2 (27), LqqIT2 (28) and LqhIT2 (19). These toxins are polypeptides of 60 to 65 amino acids which possess unique and similar primary amino acid sequences that are distinct from the excitatory toxins. These toxins induce a slow, progressive paralysis and complete relaxation of the musculature of the insect. This activity is the result of the blockage of evoked action potentials (28, 29), and is attributable to the suppression of the sodium channel conductance and depolarization of the axonal membrane.

The methods and strategies used for preparation of recombinant baculoviruses that express heterologous genes are well known in the art (6, 7, 30). These methods of gene expression have afforded economic preparation of mammalian proteins in a eukaryotic expression vector system, in many instances resulting in proteins that have achieved their proper tertiary conformation and formed the proper disulfide bridges necessary for activity.

One method for introduction of heterologous genes into the baculovirus genome is by homologous recombination between viral genomic DNA and a suitable "transfer vector" containing the heterologous gene of interest. These transfer vectors are generally plasmid DNAs that are capable of autonomous replication in bacterial hosts, affording facile genetic manipulation. Baculovirus transfer vectors also contain a genetic cassette comprising a region of the viral genome that has been modified to include the following features (listed in the 5' to 3' direction): 1) viral DNA comprising the 5' region of a non-essential genomic region; 2) a viral promoter; 3) one or more DNA sequences encoding restriction enzyme sites facilitating insertion of heterologous DNA sequences; 4) a transcriptional termination sequence; and 5) viral DNA comprising the 3' region of a non-essential genomic region. A heterologous gene of interest is inserted into the transfer vector at the restriction site downstream of the viral promoter. The resulting cassette comprises a chimeric gene wherein the heterologous gene is under the transcriptional control of the viral promoter and transcription termination sequences present on the transfer vector. Moreover, this chimeric gene is flanked by viral DNA sequences that facilitate homologous recombination at a non-essential region of the viral genome. Recombinant viruses are created by co-transfecting insect cells (capable of supporting viral replication) with viral genomic DNA and the recombinant transfer vector. Homologous recombination between the flanking viral DNA sequences present on the transfer vector and the homologous sequences on the viral genomic DNA takes place and results in insertion of the chimeric gene into a region of the viral genome that does not disrupt an essential viral function. This recombined genomic DNA is eventually packaged into an infectious recombinant virion.

In a preferred embodiment, the non-essential region of the viral genome that is present on the transfer vector comprises the region of the viral DNA responsible for polyhedrin production. Most preferred is a transfer vector that contains the entire polyhedrin gene between the flanking sequences that are involved in homologous recombination. Recombination with genomic DNA from viruses that are defective in polyhedrin production (due to a defect in the genomic copy of the polyhedrin gene) will result in restoration of the polyhedrin-positive phenotype. This strategy facilitates identification and selection of recombinant viruses.

In another embodiment, baculoviral genomic DNA can be directly modified by introduction of a unique restriction enzyme recognition sequence into a non-essential region of the viral genome. A chimeric gene comprising the heterologous gene to be expressed by the recombinant virus, operably linked to regulatory sequences capable of directing gene expression in baculovirus-infected insect cells, can be constructed and inserted directly into the viral genome at the unique restriction site. This strategy eliminates the need for construction of transfer vectors and reliance on homologous recombination for generation of recombinant viruses. This technology is described by Ernst et al. (31) and in WO94/28114 (32).

Recombinant baculovirus vectors suitable for delivery of genetically encoded insect-specific neurotoxins require optimal toxin gene expression for maximum efficacy. A number of strategies can be employed by the skilled artisan to design and prepare recombinant baculoviruses wherein toxin gene expression results in sufficient quantities of toxin produced at appropriate times during infection in a functional form, and is available for binding to target cells within the insect host.

One key to optimal gene expression is selection of an appropriate promoter element that directs transcription of the gene. Several baculovirus promoters have been described that mediate gene expression at various levels, and at different times during the viral life cycle. For instance, the polyhedrin promoter is a very strong baculovirus promoter that directs the production of the polyhedrin protein, the primary protein comprising the viral nucleocapsid. This gene is expressed late during the viral life cycle, and messenger RNA encoded by this gene can account for 20% or more of the total polyadenylated message in the infected cell. Other promoters can be chosen that are of similar strength and are expressed late in the virus life cycle, including the P10 and basic promoters. Moreover, baculoviral promoters have been described that are induced by transcription factors early during the viral life cycle (33-36), including the immediately-early promoters IE1 and IEN, the delayed-early promoter 39K or one of the promoters found on the HindIII-K fragment of the genome of AcNPV (49). These promoters may provide an additional means for accelerating the pest control capabilities of baculoviral insecticides.

Secretion of the synthesized toxin from recombinant baculovirus-infected cells is a prerequisite for more rapid onset of insecticidal effects in infected insects relative to effects induced by non-recombinant viruses (16,18). Eukaryotic proteins destined for extracellular secretion from the cell often employ short signal peptides for directing proteins to the endoplasmic reticulum. The signal peptide is then cleaved by a signal peptidase on the lumenal side of the endoplasmic reticulum membrane, and the mature protein, in this case an insect toxin, is packaged for secretion from the cell. O'Reilly et al. (6) have demonstrated that signal sequences are functional in the baculovirus

expression system. Appropriate signal sequences can be selected from the group consisting of the cuticle signal sequence from *Drosophila melanogaster*, the chorion signal sequence from *Bombyx mori*, the apolipophorin signal sequence from *Manduca sexta*, the sex specific signal sequence from *Bombyx mori*, the adipokinetic hormone signal sequence from *Manduca sexta*, the pBMHPC-12 signal sequence from *Bombyx mori*, the esterase-6 signal sequence from *Drosophila melanogaster* and the signal sequence from the viral ecdysone glucosyltransferase protein. In addition, many naturally occurring signal sequences present on heterologous eukaryotic proteins will function in baculovirus-infected insect cells to mediate extracellular secretion.

The sequence of nucleotides encoding the signal peptide and the toxin protein may also impact the quantity of toxin produced, and thereby influence the efficiency and speed with which the infected insect succumbs to treatment with engineered viruses. Deviations in the nucleotide sequence that comprise the codons encoding the amino acids of the growing toxin polypeptide chain allow for variations in the sequence coding for the gene (37). Since each codon consists of three nucleotides, and the nucleotides comprising DNA are restricted to four specific bases, there are 64 possible combinations of nucleotides, 61 of which encode amino acids (the remaining three codons encode signals ending translation). As a result, many amino acids are designated by more than one codon. For example, the amino acids alanine and proline are coded for by four triplets, serine and arginine by six, whereas tryptophan and methionine are coded by just one triplet. This degeneracy allows for DNA base composition to vary over a wide range without altering the amino acid sequence of the proteins encoded by the DNA. One hypothesis for the evolutionary development and maintenance of this degeneracy is that the flexibility that results from the ability of several codons to encode a single amino acid minimizes the potential deleterious effects of point mutations in the nucleotide sequence. Accordingly, not all point mutations will result in a codon that directs the incorporation of an unexpected amino acid and thus results in synthesis of a missense protein, or in the insertion of a translational termination codon, resulting in premature termination of translation and the production of a truncated protein.

Many organisms display a bias for use of particular codons to code for insertion of a particular amino acid in a growing peptide chain. Codon preference or codon bias, differences in codon usage between organisms, is afforded by degeneracy of the genetic code, and is well documented among many organisms (38-43). Codon bias often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, *inter alia*, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in

baculovirus expression systems based on optimization of these translational factors. Synthetic genes can thus be constructed in order to take advantage of the codon bias displayed by the virus genome. The skilled artisan appreciates the likelihood of successful gene expression if codon usage is biased towards those codons favored by the virus or the cells in which the virus replicates. Determination of preferred codons can be based on an exhaustive survey of viral and insect genes where sequence information is available. However, this information is of questionable value where the sequence information is derived from insects that are only distantly evolutionarily related to the natural host for the virus. For example, the use of codons biased on the genome of *Drosophila melanogaster* for expression of heterologous genes in AcNPV is not rational since the last common ancestor of flies (e.g., *Drosophila*) and moths (e.g., *Lepidoptera*) dates back approximately 250 million years (44, 45). More useful and compelling information for basing codon selection in favor of gene expression in NPVs can be obtained by simply examining codon bias in genes from the natural hosts of the virus (i.e., lepidopteran insects) and genes encoding highly expressed viral proteins.

Promoters directing expression of polyhedrin and P10 proteins of NPVs are known to be very late and strong promoters, and are therefore routinely chosen for directing expression of heterologous genes in baculovirus expression systems. In fact, 27 to 48 h after infection of an insect host cell by AcNPV, approximately 20% of the total polyadenylated RNA in the insect cell is polyhedrin mRNA (46, 47). In turn, the polyhedrin mRNA is efficiently translated, leading to an abundance of polyhedrin protein. Due to promoter strength and importance of the polyhedrin protein in the NPV life cycle, there has been constructed a synthetic version of the LqhIT2 gene based on the codon frequencies found in the NPV polyhedrin genes, including those from AcNPV, *Bombyx mori* NPV, *Spodoptera frugiperda* NPV, *Spodoptera littoralis* NPV and *Orgyia pseudotsugata* NPV. Codons were selected based on the frequency of appearance in these five NPV genes.

Preferred codons are easily detected when codons encoding the 20 amino acids and stop signal comprising the polyhedrin proteins are surveyed. For example, in the case of the amino acid glycine, GGC is the codon predominantly used at a frequency of 60% compared to GCA and GGG at frequencies of 14 and 4%, respectively. Other prefered codons are easily identified including: aspartic acid, GAC (74%) versus GAT (26%); arginine, CGC (37%) or CGT (33%) versus CCG (1%) or CGA (1%); asparagine, AAC (86%) versus AAT (14%); isoleucine, ATC (69%) versus ATA (6%); threonine, ACC (53%) versus ACA (4%) or ACG (13%); cystine, TGC (73%) versus TGT (27%); tyrosine, TAC (81%) versus TAT (19%); phenylalanine, TTC (65%) versus TTT (35%); glutamine, CAA (94%) versus CAG (6%); histidine, CAC (83%) versus CAT (17%); and proline, CCC (58%) versus CCA (10%). On the basis of these predominant and

distinct discrepancies in codon usage, we designed and synthesized a synthetic gene for LqhIT2.

In fact, the differences in codon usage observed in the NPV polyhedrin genes are also shared by the host cells and organisms that the viruses infect. The following Lepidopteran genes were considered:

Insect	Gene	Accession No.[1]
Heliothis virescens	Cytochrome P-450	U23506
Heliothis virescens	Juvenile Hormone Binding Protein	U22515
Heliothis virescens	Odorant Binding Protein	S62226
Heliothis virescens	Pheromone Binding Protein	S62222
Heliothis virescens	Mitochondrial p63 Chaperonin	X56034
Heliothis virescens	ATPase	L16884
Heliothis virescens	Juvenile Hormone Esterase	J04955
Trichoplusia ni	Preproattacin A	U46130
Trichoplusia ni	Lysozyme Precursor Protein	U38782
Trichoplusia ni	Cecropin A Precursor Protein	U38645
Trichoplusia ni	HSP70	U23504
Trichoplusia ni	Basic Juvenile Hormone^a Hemolymph Protein 2	L03281
Trichoplusia ni	Basic Juvenile Hormone^a Hemolymph Protein 1	L03280
Spodoptera frugiperda	Endoprotease FURIN.	Z68888
Spodoptera frugiperda	Immunophilin FKBP46	U15038

[1] GenBank

In many instances, a similar codon preference was detected between the polyhedrin genes and lepidopteran genes. For example, in the case of aspartic acid, the codon GAC is used with 74% and 64% frequency for polyhedrin (polh) and lepidopteran (lep) genes, respectively. Other preferred codon frequencies include: isoleucine, ATC-polh 69% and ATC-lep 52%; threonine, ACC-polh 53% and ACC-lep 36%; cystine, TGC-polh 73% and TGC-lep 57%; tyrosine, TAC-polh 81% and TAC-lep 73%; and phenylalanine, TTC-polh 65% and TTC-lep 73%. In addition to this observable parallelism, in no case, in the comparison of polyhedrin and lepidopteran codon frequencies, does the use of a particular codon distinctly contrast between the two gene groups. Table 1 summarizes the frequency of codon utilization deduced from observation of five polyhedrin genes of nuclear polyhedrosis viruses and fifteen lepidopteran genes.

Table 1

Frequency of Codon Utilization in NPV Polyhedrin Genes and Lepidopteran genes

		Frequency[1]	
Amino Acid	Codon	Polyhedrin	Lepidopteran
Alanine	GCA	0.08	0.19
	GCC	0.38	0.32
	GCG	0.25	0.20
	GCT	0.30	0.29
Arginine	AGA	0.12	0.17
	AGG	0.15	0.23
	CGA	0.01	0.09
	CGC	0.37	0.26
	CGG	0.01	0.08
	CGT	0.33	0.16
Asparagine	AAC	0.86	0.66
	AAT	0.14	0.34
Aspartic Acid	GAC	0.74	0.64
	GAT	0.26	0.36
Cysteine	TGC	0.73	0.57
	TGT	0.27	0.43
Glutamine	CAA	0.94	0.51
	CAG	0.06	0.49
Glutamic Acid	GAA	0.46	0.51
	GAG	0.54	0.49
Glycine	GGA	0.14	0.29
	GGC	0.60	0.26
	GGG	0.04	0.09
	GGT	0.23	0.35
Histidine	CAC	0.83	0.66
	CAT	0.17	0.34
Isoleucine	ATA	0.06	0.17
	ATC	0.69	0.52
	ATT	0.25	0.31
Leucine	CTA	0.13	0.09
	CTC	0.28	0.18
	CTG	0.27	0.27

	CTT	0.14	0.16
	TTA	0.07	0.12
	TTG	0.11	0.17
Lysine	AAA	0.46	0.36
	AAG	0.54	0.64
Methionine	ATG	1.00	1.00
Phenylalanine	TTC	0.65	0.73
	TTT	0.35	0.27
Proline	CCA	0.10	0.26
	CCC	0.58	0.26
	CCG	0.16	0.18
	CCT	0.15	0.30
Serine	AGC	0.29	0.15
	AGT	0.14	0.13
	TCA	0.06	0.17
	TCC	0.08	0.21
	TCG	0.29	0.14
	TCT	0.14	0.20
Threonine	ACA	0.04	0.23
	ACC	0.53	0.36
	ACG	0.13	0.14
	ACT	0.30	0.27
Tryptophan	TGG	1.00	1.00
Tyrosine	TAC	0.81	0.73
	TAT	0.19	0.27
Valine	GTA	0.12	0.23
	GTC	0.28	0.30
	GTG	0.43	0.24
	GTT	0.16	0.23
End	TAA	1.00	0.48
	TAG	0.00	0.33
	TGA	0.00	0.19

[1] A frequency value of 1.00 = 100%.

In some cases, alternative codons were chosen in order to facilitate genetic manipulation. The codon-biased version of the gene encoding the LqhIT2 toxin (SEQ ID NO:12) resulted in conversion of 41 of 186 nucleotides from those present in the cDNA copy of the native coding region of LqhIT2 (SEQ ID NO:11).

In order to prepare the codon-optimized LqhIT2 gene for insertion into a baculovirus vector, five sets of complimentary oligonucleotides (Figure 2; SEQ ID NO: 1-10) were designed and synthesized by standard synthetic methods, each encoding a specific region of the toxin protein. Each set of oligonucleotides, when annealed, formed a double stranded nucleic acid fragment possessing unique, single stranded extensions. The extensions were designed such that, upon incubation under appropriate conditions in the presence of a ligating enzymes, the fragments joined together in a directed, non-random fashion and resulted in a single nucleic acid fragment encoding a signal polypeptide linked to the authentic LqhIT2 toxin polypeptide. In addition, the resulting 5' and 3' ends encoded a restriction enzyme recognition site that, upon digestion with the appropriate enzyme, resulted in a DNA fragment that could be easily inserted into the insertion site of a previously prepared cloning vector. Ligated fragments were amplified by polymerase chain reaction, the amplified DNA was isolated and digested with an appropriate restriction enzyme, and the digested fragment was cloned into an intermediate plasmid vector. This intermediate vector facilitated manipulation and sequencing of the inserted toxin gene, thus facilitating confirmation of the identity of the inserted fragment, and to prepare for subsequent subcloning into an appropriate baculoviral transfer vector.

The confirmed toxin-encoding fragment was excised from the intermediate cloning vector and subcloned into a baculovirus transfer vector by standard molecular cloning techniques. In one embodiment, insertion into the transfer vector occurred at a position downstream of the baculoviral strong and late P10 promoter. This P10-LqhIT2 chimeric gene was flanked at the 5' and 3' ends by DNA sequences homologous to the regions of the baculovirus genome encoding the endogenous polyhedrin gene. Upon transformation of a suitable bacterial host, transformants were screened for proper orientation of the inserted fragments by observation of the electrophoretic migration of DNA following digestion with restriction endonucleases that cut the inserted DNA asymmetrically. A clone containing the insert in the correct orientation was chosen, and plasmid DNA of this recombinant transfer vector was prepared. In another embodiment, the synthetic LqhIT2 toxin-encoding structural gene was inserted into a baculovirus transfer vector at a position downstream of early IE1 promoter and hr5 enhancer regions. Identification of appropriate genetic constructs wherein the toxin structural gene was inserted into the early promoter transfer vector in the correct orientation relative to the promoter and enhancer element was carried out as described for the P10-LqhIT2 transfer vectors.

Introduction of the synthetic LqhIT2 gene into the baculovirus genome was accomplished by co-transfection of the transfer vector containing, *inter alia*, the chimeric signal peptide-toxin gene under the transcriptional control of either the P10 or IE1 promoters, the polyhedrin gene under the transcriptional control of the polyhedrin

promoter, and flanking baculovirus DNA, and purified, linearized genomic DNA from a polyhedrin-negative baculovirus. These DNAs were introduced into transfection-competent insect cells by techniques well known in the art. Monolayers of transfected cells were observed for plaque formation, and plaques were observed for the presence of polyhedrin inclusion bodies. The presence of inclusion bodies indicated successful recombination of the region of the transfer vector containing the polyhedrin gene and the chimeric gene encoding LqhIT2 with the polyhedrin negative baculovirus DNA. The presence of the polyhedrin gene on the transfer vector compliments the lack of a functional polyhedrin gene on the baculovirus DNA, resulting in a facile method for screening for recombinant virus.

Insertion of the LqhIT2 gene into the baculovirus genome, resulting in recombinant baculoviruses, was confirmed by Western analysis with rabbit polyclonal antibodies specific for LqhIT2 toxin. Infected cell extracts were fractionated by SDS-PAGE and subsequently transferred to a suitable medium for detection by immunoblot analysis. Surprisingly, and in contrast to data reported by others, the infected cell extracts contained a polypeptide of molecular weight similar to that expected LqhIT2 that was specifically recognized by antibodies raised against purified LqhIT2 toxin. This is the first report of successful expression of a gene encoding the LqhIT2 protein.

Recombinant baculoviruses expressing LqhIT2 were evaluated for their ability to control a target insect population by measuring survival of first or third instar larvae of *H. virescens* after oral infection with the recombinant and wild-type AcNPV. Insect larvae were fed on diet inoculated with recombinant virus expressing the LqhIT2 toxin. In addition, control insects were allowed to feed on uninoculated diet or diet containing 1) wild-type, non-recombinant virus or 2) recombinant viruses expressing AaIT, a widely employed insect-specific scorpion toxin. Larvae were monitored for behavioral changes and, ultimately, mortality. Insects feeding on diet containing AcLqhIT2 or CG201-3-1 succumbed more rapidly than those fed wild-type virus. Surprisingly, recombinant viruses expressing LqhIT2 under the transcriptional control of either the late P10 promoter or the early IE1 promoter demonstrated more rapid insecticidal effects than recombinants expressing AaIT. Insect control efficacy of AcLqhIT2 was also demonstrated in plant protection experiments. Insect larvae were inoculated with test and control viruses by feeding on diet incorporating the various viruses. Infected larvae were placed on soybean plants, and, following a suitable incubation period, destruction of plant material was quantified. Results of the experiments indicated that AcLqhIT2 treatment results in significantly less plant destruction relative to wild-type virus and recombinant viruses expressing AaIT.

Compositions of this invention will generally be used in formulation with an agriculturally suitable carrier comprising a liquid or solid diluent. Useful formulations

include dusts, granules, baits, pellets, suspensions, emulsions, wettable powders, dry flowables and the like, consistent with the physical properties of the active ingredient and compatible with the virus, mode of application and environmental factors such as soil type, moisture and temperature. Sprayable formulations can be extended in suitable media and used at spray volumes from about one to several hundred liters per hectare. High strength compositions are primarily used as intermediates for further formulation. The formulations will typically contain effective amounts of active ingredient, diluent and surfactant within the following approximate ranges which add up 100 weight percent.

	Weight Percent		
	Active Ingredient	Diluent	Surfactant
Wettable Powders	5-90	0-74	1-10
Oil Suspensions, Emulsions, Solutions, (including Emulsifiable Concentrates)	5-50	40-95	0-15
Dusts	1-25	70-99	0-5
Granules, Baits and Pellets	0.01-99	5-99.99	0-15
High Strength Compositions	90-99	0-10	0-2

Typical solid diluents are described in Watkins, et al., *Handbook of Insecticide Dust Diluents and Carriers*, 2nd Ed., Dorland Books, Caldwell, New Jersey. Typical liquid diluents and solvents are described in Marsden, *Solvents Guide*, 2nd Ed., Interscience, New York, (1950). *McCutcheon's Detergents and Emulsifiers Annual*, Allured Publ. Corp., Ridgewood, New Jersey, as well as Sisely and Wood, *Encyclopedia of Surface Active Agents*, Chemical Publ. Co., Inc., New York, (1964), list surfactants and recommended uses. All formulations can contain minor amounts of additives to reduce foam, caking, corrosion, undesired microbiological growth, and the like. Care must be taken to ensure that all ingredients of the composition are mutually compatible and do not contribute to loss of the virus infectivity.

Fine solid compositions are made by blending and, usually, grinding as in a hammer mill or fluid energy mill. Water-dispersible granules can be produced by agglomerating a fine powder composition; see for example, Cross et al., *Pesticide Formulations*, Washington, D.C., (1988), pp 251-259. Suspensions are prepared by wet-milling; see, for example, U.S. 3,060,084. Granules and pellets can be made by spraying the active material upon preformed granular carriers or by agglomeration techniques. See Browning, "Agglomeration", *Chemical Engineering*, December 4, 1967, pp 147-148, *Perry's Chemical Engineer's Handbook*, 4th Ed., McGraw-Hill, New York, (1963), pages 8-57 and following, and WO 91/13546.

For further information regarding the art of formulation, see U.S. 3,235,361, Col. 6, line 16 through Col. 7, line 19 and Examples 10-41; U.S. 3,309,192, Col. 5, line 43 through Col. 7, line 62 and Examples 8, 12, 15, 39, 41, 52, 53, 58, 132, 138 -140, 162-164, 166, 167 and 169-182; U.S. 2,891,855, Col. 3, line 66 through Col. 5, line 17 and Examples 1-4; Klingman, *Weed Control as a Science*, John Wiley and Sons, Inc., New York, (1961), pp 81-96; and Hance et al., *Weed Control Handbook*, 8th Ed., Blackwell Scientific Publications, Oxford, (1989).

In the following examples, all percentages are by weight and all formulations are prepared in conventional ways.

Example A

Wettable Powder

baculovirus	65.0%
dodecylphenol polyethylene glycol ether	2.0%
sodium ligninsulfonate	4.0%
sodium silicoaluminate	6.0%
montmorillonite (calcined)	23.0%.

Example B

Granule

baculovirus	10.0%
attapulgite granules (low volatile matter, 0.71/0.30 mm; U.S.S. No. 25-50 sieves)	90.0%.

Example C

Extruded Pellet

baculovirus	25.0%
anhydrous sodium sulfate	10.0%
crude calcium ligninsulfonate	5.0%
sodium alkylnaphthalenesulfonate	1.0%
calcium/magnesium bentonite	59.0%.

The compositions of this invention exhibit activity against a wide spectrum of foliar-feeding, fruit-feeding, stem feeding and seed-feeding lepidopterous pests important in agriculture, forestry, greenhouse crops, ornamentals, nursery crops and fiber products. Those skilled in the art will appreciate that not all compositions are equally effective against all growth stages of all pests. Nevertheless, all of the compositions of this invention display activity against larvae of the Order Lepidoptera. Specifically, the compositions are active against fall armyworm (*Spodoptera frugiperda*), tobacco budworm (*Heliothis virescens*), corn earworm (*Helicoverpa zea*), American bollworm

(*Heliothis armigera*), beet armyworm (*Spodoptera exigua*), diamondback moth (*Plutella xylostella*) and cabbage looper (*Trichoplusia ni*).

Compositions of this invention can also be mixed with one or more other insecticides, fungicides, acaricides, or other biologically active compounds to form a multi-component pesticide giving an even broader spectrum of agricultural protection. Examples of other agricultural protectants with which the recombinant baculoviruses of this invention can be formulated are insecticides that are sodium channel agonists (i.e., pyrethroids), sodium channel blocking agents (i.e., pyrazolines), acetylcholinesterase inhibitors (i.e., organophosphates and carbamates), nicotinic acetylcholine binding agents, gabaergic binding agents, octapine agonists or antagonists (i.e., formamidines) and oxyphos uncouplers (i.e., pyrrole insecticides). Specific examples of insecticides that can be mixed with the recombinant baculoviruses of this invention are: avermectin B, monocrotophos, tetrachlorvinphos, malathion, parathion-methyl, diazinon, profenofos, sulprofos, triflumuron, diflubenzuron, methoprene, buprofezin, thiodicarb, acephate, azinphosmethyl, chlorpyrifos, dimethoate, fipronil, flufenprox, fonophos, isofenphos, methidathion, metha-midophos, phosmet, phosphamidon, phosalone, pirimicarb, phorate, terbufos, trichlorfon, methoxychlor, bifenthrin, biphenate, tefluthrin, fenpropathrin, fluvalinate, imidacloprid, metaldehyde and rotenone. In addition, fungicides such as carbendazim, thiuram, dodine, maneb, chloroneb, benomyl, cymoxanil, fenpropidine, fenpropimorph, triadimefon, captan, thiophanate-methyl, thiabendazole, phosethyl-Al, chlorothalonil, dichloran, metalaxyl, captafol, iprodione, oxadixyl, vinclozolin, kasugamycin, myclobutanil, tebuconazole, difenoconazole, diniconazole, fluquinconazole, ipconazole, metconazole, penconazole, propiconazole, uniconzole, flutriafol, prochloraz, pyrifenox, fenarimol, triadimenol, diclobutrazol, copper oxychloride, furalaxyl, folpet, flusilazol, blasticidin S, diclomezine, edifenphos, isoprothiolane, iprobenfos, mepronil, neo-asozin, pencycuron, probenazole, pyroquilon, tricyclazole, validamycin, and flutolanil can also be mixed with the recombinant baculoviruses of this invention.

In certain instances, combinations with other insecticides having a similar spectrum of control but a different mode of action will be particularly advantageous for resistance management.

Lepidopterous pests are controlled and protection of agronomic, horticultural and specialty crops, animal and human health is achieved by applying one or more of the compositions of this invention, in an effective amount, to the environment of the pests including the agronomic and/or nonagronomic locus of infestation, to the area to be protected, or directly on the pests to be controlled. A preferred method of application is by spraying. Alternatively, granular formulations of these compounds can be applied to the plant foliage or the soil. Other methods of application include direct and residual

sprays, aerial sprays, seed coats, microencapsulations, systemic uptake, foggers, aerosols, dusts and many others. The compositions can be incorporated into baits that are consumed by the insects or in devices such as traps and the like.

The compositions of this invention can be applied in their pure state, but most often application will be of a formulation comprising the instant baculoviruses with suitable carriers, diluents, and surfactants and possibly in combination with a food (bait) depending on the contemplated end use. A preferred method of application involves spraying a water dispersion or refined oil suspension of the arthropodicides. Combinations with spray oils, spray oil concentrations, spreader stickers, adjuvants, solvents, and synergists often enhance arthropodicidal efficacy.

The rate of application required for effective control will depend on such factors as the species of insect to be controlled, the pest's life cycle, life stage, its size, location, time of year, host crop or animal, feeding behavior, mating behavior, ambient moisture, temperature, and the like. Under normal circumstances, application rates of about 0.01 to .05 kg of active ingredient per hectare are sufficient to control pests in agronomic ecosystems, but as little as 0.001 kg/hectare may be sufficient or as much as 1 kg hectare may be required. For nonagronomic applications, effective use rates will range from about 1.0 to 50 mg/square meter but as little as 0.1 mg/square meter may be sufficient or as much as 150 mg/square meter may be required.

EXAMPLE 1

Construction of the Synthetic LqhIT2 Structural Gene

In order to prepare the NPV-biased form of the LqhIT2 gene, ten oligonucleotides were designed (Figure 2; SEQ ID NO:1-10) and synthesized by standard phosphoramadite chemistry. These oligonucleotides were phosphorylated using Gibco/BRL (Gaithersburg, MD) kinase, annealed, and ligated using Gibco/BRL ligase following the scheme depicted in Figure 3, and employing the manufacturer's recommended protocols. Ligated fragments were then amplified by employing the polymerase chain reaction (PCR) using Perkin-Elmer Cetus AmpliTaq™ Polymerase (Norwalk, CT) according to the manufacturer's protocol and the modifications described below. Oligonucleotide Lq1 (SEQ ID NO:1) was used as the forward primer and oligonucleotide Lq10 (SEQ ID NO:10) served as the reverse primer. The descriptions of these protocols are set out below in greater detail.

Ten separate phosphorylation reactions (one for each oligonucleotide) were carried out. Two-hundred and fifty pmol of each oligonucleotide (SEQ ID NO:1-10) were placed in a 1.5 mL microcentrifuge tube. Five uL of 10x kinase buffer, 1 uL of 1 mM ATP, 6 uL of kinase (Gibco/BRL, 10 units/uL), and a sufficient volume of water was added to each tube in order to bring the total reaction volume to 50 uL. The ten tubes were incubated at 37°C for 1 h. Following incubation, five uL of each phosphorylated

oligonucleotide (25 pmol) was placed into a single microcentrifuge tube, and the tube was placed into a dry heat blockset at 95°C. The heat block was then turned off and allowed to cool to room temperature to facilitate annealing of phosphorylated oligonucleotides. Fifty uL of the mixture of phosphorylated, annealed oligonucleotides were placed into a separate microcentrifuge tube along with 15 uL of 5X ligase buffer, 3 uL of 10 mM ATP, 4 uL of ligase enzyme (Gibco/BRL, 5 units/uL) and 3 uL of deionized water. This tube was incubated at 37°C for 30 min and subsequently stored at room temperature overnight.

The synthetic nucleic acid fragment comprising the annealed and ligated oligonucleotides was amplified by PCR. Three PCR reactions were performed on varying dilutions of template DNA (comprising the phosphorylated, annealed and ligated oligonucleotides). The following reaction mix was employed for PCR reactions:

61.5 uL of deionized water
10 uL of 10X PCR buffer (Perkin-Elmer Cetus)
2 uL each of dATP, dCTP, dGTP, dTTP (200 uM each)
0.5 uL of Ampli Taq™ Polymerase (2.5 units/100 uL)

Template DNA was diluted 1:100, 1:1,000 and 1:10,000 (v/v) with deionized water. Eighty uL of the reaction mix was placed in each of three 0.5 mL microcentrifuge tubes. Five uL (100 pmol) of oligonucleotide Lq1 (SEQ ID NO:1) and 5 uL (100 pmol) of oligonucleotide Lq10 (SEQ ID NO:10), (serving as the forward and reverse PCR primers) respectively, was added to each tube. Ten uL of appropriately diluted template was added to each tube. PCR reactions were carried out using a Perkin-Elmer DNA Thermocycler® programmed to carry out the following amplification protocol:

STEP 1	96°C	3 minutes
1 cycle	75°C	3 minutes
STEP 2	95°C	30 seconds
25 cycles	75°C	2 minutes
STEP 3	95°C	30 seconds
1 cycle	75°C	5 minutes

Products resulting from amplification were analyzed by electorphoresis through a 2% agarose gel. An amplified fragment of approximately 300 base pairs was observed for each reaction.

Following PCR amplification of the LqhIT2 gene and flanking regions, the 300 bp band was isolated from a 1.2 % agarose gel and purified using a SpinBind DNA recovery

system (FMC, Rockland, ME) according to the manufacturer's protocol. The isolated fragment was digested with BamHI in order create cohesive 5' and 3' ends of the synthetic oligonucleotide containing the LqhIT2 gene and signal sequence. The digested fragment was then inserted into the pTZ-18R plasmid (Pharmacia, Piscataway, NJ) at the BamHI cloning site using standard molecular cloning techniques. Following transformation of pTZ-18R into *E. coli* XL1 Blue (Stratagene, Menasha, WI), isolated colonies were chosen and plasmid DNA was prepared. Eight positive clones were identified and sequenced with the commercially available forward and reverse primers of PTZ-18R (Pharmacia). One clone (No. 16) was found to contain the correct sequence encoding for synthetic gene and signal sequence. The resulting plasmid contained two BamHI restriction sites: one site near the 5' end of the toxin gene and the other site following the stop codon. Plasmid DNA was prepared according to standard protocols, and was digested with BamHI to release the inserted 300 base pair fragment containing the LqhIT2 gene and signal sequence. This fragment was separated from vector sequences by electrophoresis through a 1.6 % agarose gel, excision of the band corresponding to this fragment and purification by the SpinBind™ DNA recovery method (FMC).

EXAMPLE 2

Construction and Testing of Recombinant AcNPV Comprising the Synthetic LqhIT2 Structural Gene Under the Transcriptional Control of a Baculovirus Late Promoter

The purified LqhIT2 gene fragment was inserted into the BglII cloning site of the baculovirus transfer vector pAcUW21 (Pharmingen, San Diego, CA) by standard molecular cloning techniques. This subcloning resulted in insertion of the synthetic gene on the 3' side of the vector-borne P10 promoter, and on the 5' side of a non-functional *lac*Z fragment resident on the transfer vector. Following ligation, DNA was transformed into the *E. coli* XL1 Blue (Stratagene). As a result of the ligation, both the BglII and BamHI sites were destroyed, and resulting plasmids were screened for a unique asymmetric SphI site located at the 3' end of the LqhIT2 gene. Three positive clones were identified from electrophoretic analysis of plasmid DNA isolated from twenty-three transformants. The direction of insertion was confirmed by analysis of plasmid digested simultaneously with SphI (cuts at the 3' end of the LqhIT2 gene) and BamHI (cuts within the coding sequence of the polyhedrin gene present on the transfer vector). One clone was identified as carrying the LqhIT2 gene in the correct orientation. This construction resulted in the LqhIT2 synthetic structural gene inserted downstream of the P10 promoter and upstream of the polyhedrin gene (pAcUW21.LqhIT2; see Fig. 5) .

Spodoptera frugiperda cells (Sf-9) were propagated in ExCell™ 401 media (JRH Biosciences, Lenexa, KS) supplemented with 3.0 % fetal bovine serum.

Lipofectin™ (50 µL at 0.1 mg/mL, Gibco/BRL) was added to a 50 µL aliquot of pAcUW21.LqhIT2 (500 ng) and linearized polyhedrin-negative AcNPV (2.5 µg, Baculogold™ viral DNA, Phamingen). Sf-9 cells (approximate 50% monolayer) were co-transfected with the viral DNA/transfer vector solution. The supernatant fluid from the co-transfection experiment was collected at 5 days post-transfection and recombinant viruses were isolated employing standard plaque purification protocols, wherein only polyhedrin-positive plaques were selected (12).

A total of seven plaques were isolated; each was suspended in 500 µL of ExCell™ media supplemented with 2.5% fetal bovine serum. Sf-9 cells in 35 mM petri dishes (50% monolayer) were inoculated with 100 µL of the viral suspension, and supernatant fluids were collected at 5 days post infection. In order to prepare larger quantities of virus for characterization, these supernatant fluids were used to inoculate larger tissue cultures for large scale propagation of recombinant viruses.

Insertion of the LqhIT2 gene into the baculovirus genome was confirmed by immunoblot analysis and bioassay. Sf-9 cells (50 mL) were infected with wild-type AcNPV (control) or AcLqhIT2 (5 individual isolates). Three days post infection, infected cells were collected and growth media was removed by centrifugation of cell suspensions. Spent culture media was decanted and the remaining cells were lysed with a Branson Sonifier™ (Model 450) for 30 s at setting 2. Cellular debris was then removed by centrifugation at 15,000 rpm for 10 min in a refrigerated microcentrifuge (4°C).

Protein concentrations of cell infected cell sonicates were quantified using the BCA Protein Assay (Pierce, Rockford, IL) according to the manufacturer's instructions. A standard curve was prepared based on known concentrations of Bovine Serum Albumin. Samples and standards were incubated for 30 min at 37°C, and the absorbence at 562 nm was measured with a spectrophotometer. Protein concentrations were determined for each sample by linear regression analysis.

Individual proteins in quantified samples were separated by protein electrophoresis. Samples were diluted to 3-4 mg/mL protein with deionized water. Twenty-five µL of each sample was added to 75 µL of electrophoresis sample buffer (3.8 mL deionized H_2O, 1.0 mL 0.5 M Tris-HCl, pH 6.8, 0.8 mL glycerol, 1.6 mL 10% (w/v) SDS, 0.4 mL ß-mercaptoethanol, 0.4 mL 0.5% bromophenol blue). Molecular weight standards were prepared by diluting 1 µl of biotinylated protein molecular weight standards (Gibco) in 100 µL of sample buffer. Samples and standards were then heated at 95°C for 2 min. Samples were loaded (20 µl/well) onto a 15% Mini-Protean II Tris-HCl Ready Gel (Bio-Rad, Melville, NY). Electrophoresis running buffer (3 g Tris base, 14.4 g glycine, 1.0 g SDS in 1 L deionized H_2O) was added to the assembled electrophoresis gel apparatus and samples were electrophoresised for approximately 1.5 h at 40 mA.

Following electrophoresis, separated proteins were transferred from the electrophoretic gel to a nitrocellulose filter by western blotting. Blotter paper (3MM; Schleicher and Schuell, Keene, NH) and nitrocellulose (BA-S NC; Schleicher and Schuell) were cut to the approximate dimensions of the gel and soaked in western transfer buffer (11.6 g Tris base, pH 8.3, 5.8 g glycine, 0.74 g SDS, 400 mL methanol, 1.6 L deionized H_2O). The blotter paper, nitrocellulose and gel were assembled in the following sequence: 3 sheets of blotter paper, gel, nitrocellulose, and 3 pieces of blotter paper. This "sandwich" was placed in the transfer apparatus such that the gel was oriented towards the cathode and the nitrocellulose membrane towards the anode. Proteins were transferred by applying a current to the transfer apparatus for 4 h at 60 mA. Following transfer, the apparatus was disassembled and the nitrocellulose filter was allowed to air dry.

Immunoblot analysis of transferred proteins proceeded by the following steps, all of which were performed on a rotary shaker at room temperature. Sites on the nitrocellulose membrane that were unoccupied by transferred proteins were blocked with 3% (w/v) gelatin dissolved in TTBS (20 mM Tris, 500 mM NaCl, pH 7.5, 0.05% Tween-20) by incubation for 30 min. The blocking solution was removed and the membrane was rinsed in 100 mL TTBS for 5 min. LqhIT2-specific rabbit polyclonal antibody (obtained from Dr. Bruce Hammock, U.C. Davis, Davis, CA) was prepared by adding 10 µL rabbit antibody to 10 mL TTBS. This primary antibody solution was applied to the blocked nitrocellulose filter and incubated for 1 h. Following this incubation, the nitrocellulose filter was washed in 3 changes of 100 mL TTBS, each wash lasting 10 min. The secondary antibody was prepared by adding 10 µL peroxidase conjugated goat antirabbit IgG (Sigma, St. Louis, MO) and 10 µL peroxidase-labeled streptavidin to 20 mL TTBS. The nitrocellulose filter was incubated with this solution for 1 h. Following this incubation, the filter was washed in 3 changes of 100 mL TTBS, each wash lasting 10 min. Detection reagent (ECL Detection kit; Amersham, Arling Heights, IL) was applied to the nitrocellulose and incubated for 60 s. The detection reagent was drained from the filter, and the filter was covered with Saran Wrap. Signal from the nitrocellulose filter was detected by exposing the processed membrane to X-ray film (Kodak X-OMAT AR, Rochester, NY) for 2 - 10 s. All five AcLqhIT2 isolates provided a positive immunoblot response near 7,000 Mr, whereas no signal was detected for AcAaIT.

Five independent AcLqhIT2 isolates were screened for biological activity. This assay involved comparison of biological activity of AcLqhIT2 recombinant viruses to wild-type AcNPV (control) and recombinant AcNPVs expressing the AaIT toxin. Third instar larvae of *H. virescens* were infected orally by consumption of diet that contained test and control viruses, and monitored for behavioral changes and mortality.

Cells infected with AcLqhIT2, AcNPV and AcAaIT were harvested, and PIBs were released by consecutive washes with 0.5 % w/v sodium dodecyl sulfate, 5M NaCl, and finally deionized water. After the water wash, freed PIBs were suspended in a small volume of deionized water and enumerated by hemocytometer counting. It was noted that significantly less PIBs and cellular debris were produced from Sf-9 cells infected with AcLqhIT2 three to four days post infection than from cells infected with wild-type AcNPV and AcAaIT.

Isolated plugs of a standard insect diet were inoculated with approximately 5000 PIBs. Individual larvae that had not fed for 12 h prior to beginning of the bioassay were allowed to consume the diet for 24 h. The larvae were transferred to an individual well in a diet tray and monitored for symptoms and mortality on a daily basis. These initial bioassays demonstrated the induction of paralysis after consumption of diet containing AcLqhIT2 and AcAaIT viruses. Surprisingly, AcLqhIT2 appeared to kill larvae more rapidly than either wild-type AcNPV or AcAaIT viruses. Three of the AcLqhIT2 isolates tested and the best AcAaIT virus were chosen for subsequent testing. Once again, the results indicated that the AcLqhIT2 viruses resulted in more rapid mortality than either of the controls.

A thorough evaluation of the insecticidal effects of the recombinant viruses was undertaken. Earlier observations indicated that the expression of LqhIT2 by AcNPV produces a cytotoxic effect in insect cells. Moreover, due to this cytotoxic effect, the yield of PIBs/cell for AcLqhIT2 is significantly reduced in comparison to wild-type AcNPV and AcAaIT. In fact, cell cultures infected with AcLqhIT2 yielded average PIB counts of 5.03×10^7/100 mL of cell culture media compared to $>1.9 \times 10^9$ PIBs/100 mL of cell culture media for AcAaIT and wild-type AcNPV. In order to prepare even larger quantities of AcLqhIT2 recombinant PIBs, viruses were propagated *in vivo*. To acquire *in vivo* produced PIBs of AcLqhIT2, AcAaIT and wild-type AcNPV, the dead insects from preliminary bioassays were processed by homogenization in deionized water, followed by probe sonification and filtration to remove cellular debris. PIBs were then enumerated using a standard hemocytometer.

Comprehensive analysis of virus-induced insect mortality was performed in experiments wherein third instar larvae of *H. virescens* were infected with test and control viruses and observed for onset of mortality. A probit analysis program (48) was used to derive time-mortality and dose-mortality curves (Table 2), and to test for significant differences in bioactivity of the recombinant (AcLqhIT2 and AcAaIT) and wild-type AcNPVs. Lethal times (LTs) were derived by allowing starved insects to feed for 24 h on diet plugs that were inoculated with 2500 PIBs per plug. These insects were then transferred to individuals wells of diet and were routinely monitored (at least twice a day) for death and/or paralysis. The approximate LT_{50} (time to kill 50% of the exposed

insects; 8 replicates, 16 larvae per replicate, n = 128 insects per treatment) for AcLqhIT2, AcAaIT and wild-type AcNPV were 82.7, 97.9 and 118 h post infection, respectively. The likelihood-ratio test (equal slopes and intercepts, C.I. = 0.95) demonstrated a significant difference among treatments: recombinant viruses, AcLqhIT2 and AcAaIT, had significantly lower LT values than larvae infected with wild-type AcNPV. Furthermore, a direct comparison of slopes and intercepts (C.I. = 0.95) of the AcLqhIT2 and AcAaIT recombinant viruses confirmed that the LT_{50} values for AcLqhIT2 were significantly lower than those for AcAaIT.

In a second set of experiments, lethal doses (LDs) were determined. Third instar larvae of *H. virescens* were fed on diets inoculated with AcLqhIT2, AcAaIT, wild-type AcNPV, and uninoculated plugs. Molten diet was prepared with varying doses of virus in order to establish dose-response curves for the sample groups. PIB concentrations of the diet plugs ranged in logarithmic dose from $1x10^2$ through $1x10^4$ PIBs per mL of diet. The test was monitored for mortality between at 94 and 160 h post feeding. The approximate LD_{50}s (number of PIBs required to kill 50% of the exposed insects; 4 replicates, 25 larvae per replicate, n = 100 insects per treatment) for AcLqhIT2, AcAaIT and wild-type AcNPV were $8.29x10^2$, $9.15x10^2$ and $1.19x10^3$ PIB/mL, respectively. The likelihood-ratio test (equal slopes and intercepts, C.I. = 0.95) showed no significant difference between the potency of the three viral treatments (Table 2).

Table 2

Lethal Doses and Times of Recombinant Viruses on 3rd Instar Larvae of *H. virescens*

Virus	LD (PIBs x 10^2/mL)			LT (hours)		
	10	50	90	10	50	90
AcLqhIT2	1.03	8.29	66.6	66.8[a]	82.7[a]	102[a]
AcAaIT	1.55	9.15	53.9	76.7[b]	97.9[b]	125[b]
Wild-type AcNPV	2.52	11.9	56.1	95.6[c]	118[c]	145[c]

a, b, c Significantly different from other treatments - POLO probit analysis program (C.I. 0.95).

PIBs = polyhedrin inclusion bodies.

LD = lethal dose.

LT = lethal time.

Further evaluation of efficacy of the viruses was conducted on two week-old soybean (Williams) plants potted in 4 inch square pots filled with METROMIX 350 growth medium (Grace-Sierra, Milpitas, CA). These plants were grown in a greenhouse at 26°C. Second instar larvae of *H. virescens* were exposed for 24 h to the viral treatments which were incorporated into the diet at a concentration calculated to result

in fatal infection of greater than 99% of treated insects. Following this exposure period, infected insects were transferred to the plants. The plant set-up consisted of one soybean plant enclosed in a clear plastic tube with an open top. A layer of white silica sand was placed on top of the potting media so fallen insects could be easily identified. Three treated insects were placed on the plant and the unit top was closed. Ten plants were used per treatment.

The test units were held in a plant holding room on a 16 h light and 8 h dark cycle and watered as needed. At the conclusion of the testing period (death of all treated insects) the test units were disassembled and plant material was quantified, employing a Li-Cor leaf area meter (Li-Cor, Lincoln, NE). The average plant area remaining was 154, 199, 223 cm^2 for wild-type AcNPV, AcAaIT and AcLqhIT2, respectively, while untreated (controls) plants had an average of 243 cm^2 of material. These data indicate 63.6, 82.2 and 92.1 percent plant protection for wild-type AcNPV, AcAaIT and AcLqhIT2, respectively, when compared to uninfested plants (Figure 7). Clearly, the recombinant viruses expressing the toxins provide increased protection, and the AcLqhIT2 provides significantly greater protection than AcAaIT.

EXAMPLE 3

Construction and Testing of Recombinant AcNPV Comprising the Synthetic LqhIT2 Structural Gene Under the Transcriptional Control of a Baculovirus Early Promoter

In the same manner as described in Example 2, the purified LqhIT2 structural gene gene fragment from Example 1 was inserted into the BglII cloning site of the baculovirus transfer vector pAcP+IE1TV3 (provided by Dr. Donald Jarvis, Texas Agricultural Experiment Station, Texas A&M University, College Station, TX). This plasmid transfer vector is a derivative of the baculovirus early promoter transfer vectors described in U.S. Pat. No. 5,162,222, incorporated herein by reference. This construct resulted in the insertion of the synthetic structural gene encoding the LqhIT2 toxin downstream of the baculovirus early IE1 promoter and the hr5 enhancer region. Following ligation, plasmid DNA was transformed into the *E. coli* XL1 Blue (Stratagene). As a result of the ligation, both the BglII and BamHI sites were destroyed, and resultant transformants were screened for possession of plasmids that contained a unique asymmetric SphI site located at the 3' end of the LqhIT2 gene.

Twenty-three SphI-positive clones were identified from electrophoretic analysis of plasmid DNA isolated from twenty-four transformants. The direction of insertion was confirmed by analysis of plasmid digested simultaneously with SphI (cuts at the 3' end of the LqhIT2 gene) and StuI (cuts within the coding sequence of the multiple clonining site present in the transfer vector downstream of the BglII cloning site). Eight clones were analyzed, four of which possessed the LqhIT2 gene in the correct orientation relative to

the direction of transcription from the early IE1 promoter/hr5 enhancer regions. DNA from one of the clones, CG201-3, was isolated and utilized to create recombinant baculovirus expression vectors by co-transfection, essentially following the protocol described in Example 2. Plaques were isolated and propagated in tissue culture, and insecticidal activity was determined by bioassay, following the protocols described in Example 2.

Neonate larvae of *H. virescens* were infected with test and control viruses and observed for the onset of mortality. For this set of bioassays, the virus was incorporated into the insect diet at a concentration of 1.0×10^4 PIBs/mL. A probit analysis program (48) was used to derive time-mortality curves and to test for signficant differences in bioactivty of the recombinant viruses (CG201-3-1 and AcLqhIT) and wild-type AcNPV. The approximate LT_{50} (time to mortality for 50% of the treated insects) for CG201-3-1, AcLqhIT and wild-type AcNPV were 44.4, 61.5 and 91.0, respectively (4 replicates, 25 larvae per replicate, n = 100 insects per treatment; Table 3). The likelihood ratio test (C.I. = 0.95) demonstrated a significant difference among treatments, indicating that application of either of the recombinant viruses resulted in more rapid mortality than treatment with wild-type AcNPV. More specifically, CG201-3-1 had significantly lower LT values than AcLqhIT, indicating that this recombinant virus kills treated insects more rapidly. This data demonstrates that utilization of an early promoter to drive expression of a codon-biased, synthetic structural gene encoding an insect-specific toxin reduces the time to mortality by 27.8 % compared to viruses where expression of an identical structural gene is controlled by a late promoter. Moreover, these data indicate that viruses expressing the synthetic toxin gene under the transcriptional control of an early promoter reduce the time to mortality of treated insect larvae by greater than 50 % when compared to treatment with non-recombinant, wild-type AcNPV.

Table 3

Lethal Times of of Recombinant Viruses

on 1st Instar Larvae of *H. virescens*

Virus	LT (hours)		
	10	50	90
CG201-3-1	32.7[a]	44.4[a]	60.3[a]
AcLqhIT	47.4[b]	61.5[b]	79.6[b]
Wild-type AcNPV	75.9[c]	91.0[c]	109[c]

a, b, c Significantly different from other treatments - POLO probit analysis program (C.I. 0.95).

LT = lethal time.

EXAMPLE 4

Construction and Testing of a Recombinant AcNPV Comprising a Synthetic LqhIT2 Structural Gene Based on the Complimentary DNA of the LqhIT2 Gene Isolated from the Scorpion, *Leiurus quinquestriatus hebraeus*

In order to evaluate increased efficacy provided by the NPV-codon biased LqhIT2 gene versus the cDNA version, the cDNA gene was synthesized and incorporated into the AcNPV viral genome by methods analogous to those described in Examples 1, 2 and 3.

The synthetic cDNA LqhIT2 gene was generated, isolated, subcloned and sequenced in a similar manner to the codon biased LqhIT2 gene described in Example 1. In order to prepare the cDNA form of the LqhIT2 gene, ten oligonucleotides were designed and synthesized by standard phosphoramadite chemistry. These oligonucleotides were phosphorylated using Gibco/BRL (Gaithersburg, MD) kinase, annealed, and ligated using Gibco/BRL ligase following the scheme depicted in Figure 3, and employing the manufacturer's recommended protocols. Ligated fragments were then amplified by polymerase chain reaction (PCR) using Perkin-Elmer Cetus AmpliTaq Polymerase (Norwalk, CT) according to the manufacturer's protocol and the modifications described below.

After PCR amplification, the gene product was cloned into the pCRTMII Vector according to the protocol provided in the Original TA Cloning Kit (Invitrogen, San Diego, CA). Following ligation, transformation and restriction map analysis, several clones were sequenced and a clone was confirmed to encode for the bombyxin signal sequence and the cDNA version of the LqhIT2 gene. The gene was inserted into the SacI/NotI cloning sites of the baculovirus transfer vector pAcP+IE1TV3. Recombinant AcNPVs were constructed using the protocols according to details described in Example 2.

Following the propogation of five candidate recombinant viruses encoding the cDNA LqhIT2 gene (IC735), the viruses were tested for efficacy, and the best viral isolate with respect to lethal time values was selected. Lethal time characterization was performed on neonate larvae, and third and fourth instar larvae of *H. virescens* for the codon optimized AcLqhIT2 (CG201-3-1), the cDNA version of AcLqhIT2 (IC735-1), and wild-type AcNPV (IC200-27) to determine any significant differences in time to kill (Table 4).

For third and fourth instar larvae, a diet pill assay was utilized to determine LT_{50} values. Stock preparations of viruses were quantified using a Reichert-Jung bright-line hemacytometer. Viral suspensions were formulated in deionized water to a final concentration of 1000-2000 PIBs/uL. Individual 50 uL diet pills were placed in each well of a sixteen-well polystyrene bioassay tray. A dose of 5000 OBs was applied to the

surface of each 50 uL diet pill, and the viral treatment was allowed to dry. One larva was then placed into each well; wells were sealed and incubated at 28°C. Upon complete consumption of the diet pill (approx. 24hrs), the larvae were transferred to untreated insect media and evaluated for mortality twice daily.

For neonate larvae, a diet incorporation assay was utilized to determine LT_{50} values. Stock virus preparations were quantified using a Reichert-Jung bright-line hemacytometer and diluted with deionized water to a concentration of 1.0×10^5 PIBs/mL. A final 1:10 dilution was performed in insect media. This virus-containing media was blended for 30 seconds to ensure a homogenous suspension of viral occlusion bodies at 1.0×10^4 PIBs/mL. The media was then poured into cells of polystyrene trays, each tray containing 25 cells. Approximately 2 mL of diet was applied to each cell. Upon solidification of the inoculated media, one neonate *H. virescens* larva was placed into each cell. Cells were sealed and incubated at 28°C. Evaluations for larval mortality were performed twice daily.

Data from these experiments was analyzed using the Vistat statistical analysis package (50). For all three larval stages tested, the CG201-3-1 encoding the codon-biased LqhIT2 gene killed insect larvae more rapidly than IC735-1 (the cDNA version of LqhIT2), and IC200-27 (wild-type AcNPV). The reduction in LT_{50} values for the codon optimized CG201-3-1 compared to IC735-1 was statistically significant for third and fourth instar larvae of *H. virescens*, with a reduction in time to kill of 22% and 9%, respectively (Table 4). These differences were even more dramatic at the LT_{90}s for neonate and third instar larvae, with approximately 15% and 32% reduction in time to kill, respctively, with the codon optimized construct, CG201-3-1 (48).

Table 4

Lethal Times (LT_{50}) of Recombinant or Wild-type Viruses on Neonate and Selected Instars of Larvae of *H. virescens*

Virus	LT_{50} (hours)		
	Neonate	Third Instar	Fourth Instar
CG201-3-1	61.7	62.9	69.3
IC735-1	65.0	81.0	76.1
Wild-type AcNPV	102	113	111

References

(1) Granados, R. R.; Lawler, K. A. *Virology* (1981), *108*, 297-308.

(2) Vlak, J. M.; Rohrmann, G. F. In *Viral Insecticides for Biological Control*, Maramorosch, K.; Sherman, K. E., Eds.; Academic Press, New York, NY. (1985), 489-542.

(3) Carbonell, L. F.; Klowden, M. J; Miller, L. K. *J. Virol.* (1985), *56*, 153-160.

(4) Carbonell, L. F.; Miller, L. K. *Appl. Environ. Microbiol.* (1987), *53*, 1412-1417.

(5) Brusca, J.; Summers, M; Couch, J.; Courtney, L. *Intervirol.* (1986), *26*, 207-222.

(6) O'Reilly, D. R.; Miller, L. K.; Luckow, V. A. (1992), *Baculovirus Expression Vectors: A Laboratory Manual*, W. H. Freeman and Company, New York.

(7) King, L. A.; Possee, R. D. (1992), *The Baculovirus Expression System*, Chapman and Hall, London.

(8) O'Reilly, D. R.; Miller, L. K. *J. Virol.* (1990), *64*, 1321-1328.

(9) O'Reilly, D. R.; Miller, L. K. *Science* (1989), *245*, 1110-1112.

(10) O'Reilly, D. R.; Miller, L. K., *Biotechnol.* (1991), *9*, 1086-1089.

(11) Carbonell, L.F.; Hodge, M.R.; Tomalski, M.D.; Miller, L.K. *Gene* (1988), *73*, 409-418.

(12) Merryweather, A. T.; Weyer, U.; Harris, M. P. G.; Hirst, M.; Booth, T.; and Possee, R. D. *J. Gen. Virol.* (1990), *71*, 1535-1544.

(13) Martens, J. W. M.; Honee, G.; Zuidema, D.; Van Lent, J. W. M.; Visser, B; Vlak, J. M. *Appl. Environ. Microbiol.* (1990), *56*, 2764-2770.

(14) Tomalski, M. D.; Miller, L. K. *Nature* (1991), *352*, 82-85.

(15) Tomalski, M. D.; Miller, L. K. *Biotech.* (1992), *10*, 545-549.

(16) Maeda, S.; Volrath, S. L.; Hanzlik, T. N.; Harper, S. A.; Maddox, D. W.; Hammock, B. D.; Fowler, E. *Virol.* (1991), *184*, 777-780.

(17) Stewart, L. M. D.; Hirst, M.; Ferber, M. L.; Merryweather, A. T.; Cayley, P. J.; Possee, R. D. *Nature* (1991), *352*, 85-88.

(18) McCutchen, B. F.; Choudary, P. V.; Crenshaw, R.; Maddox, D.; Kamita, S. G.; Palekar, N.; Volrath, S.; Fowler, E.; Hammock, B. D.; Maeda, S. *Biotech.* (1991), *9*, 848-852.

(19) Zlotkin, E.; Eitan, M.; Bindokas, V. P.; Adams, M. E.; Moyer, M.; Burkhart, W.; Fowler, E. *Biochemistry* (1991), *30*, 4814-4821.

(20) Zilberberg, N.; Zlotkin, E.; Gurevitz, M. *Insect biochem. Molec. Biol.* (1992), *22*, 199 203.

(21) Caruthers, M., in *Methodology of DNA and RNA Sequencing*, (1983), Weissman (ed.), Praeger Publishers, New York, Chapter 1.

(22) Zlotkin, E. *Phytoparasitica* (1991), *19*, 177-182.

(23) Walther, D.; Zlotkin, E.; Rathmayer, E. *J. Insect Physiol.* (1976), *22*, 1187-1194.

(24) Zlotkin, E. in *Neuropharmacology and Pesticide Action*; (1986), Ford, Lunt, Raey & Usherwood (eds.), Ellis Horwood, England, 352-383.

(25) Zlotkin, E.; Fishman, L.; Gordon, D. in *Neurotox'88: Molecular Basis of Drug and Pesticide Action*, (1988), Lunt (ed.) 35-47.

(26) Zlotkin, E.; Rochat, H.; Kupeyan, C.; Miranda, F.; Lissitzky, S. *Biochimie* (Paris) (1971), *53*, 1073-1078.

(27) Lester, D.; Lazarovici, P.; Pelhate, M.; Zlotkin, E. *Biochem. Biophys. Acta.* (1982), *701*, 370-381.

(28) Zlotkin, E.; Kaduri, D.; Gordon, D.; Pelhate, M.; Martin, M.; Rochat, H. *Arch. Biochem. Biophys.* (1985), *240*, 877-887.

(29) Zlotkin, E.; Gurevitz, M.; Fowler, E.; Adams, M. *Arch. of Insect Biochem. Physiol.* (1993), *22*, 55-73.

(30) U.S. Pat. No. 4,745,051.

(31) Ernst, W.; Grabherr, R.; Katinger, H. *Nuc. Acid Res.* (1994), *22*, 2855-2856.

(32) Patent Cooperation Treaty, Publication Number WO 94/28114.

(33) Jarvis, D.; Fleming, J.; Kovacs, G.; Summers, M.; Guarino, L. *Biotechnol.* (1990), *8*, 950-955.

(34) O'Reilly, D.; Passarelli, L.; Goldman, I.; Miller, L. *J. Gen. Virol.* (1990), *71*, 1029-1037.

(35) Guarino, L.; Summers, M.; *J. Virol.* (1987), *61*, 2091-2099.

(36) Crawford, A.; Miller, L. *J. Virol.*, (1988), *62*, 2773-2781.

(37) Alberts et al., *Molecular Biology of the Cell*, Garland, New York, pp 106-111.

(38) Grantham, R. et al. *Nucl. Acids Res.* (1980), *8*, 49-62.

(39) Grantham, R. et al. *Nucl. Acids Res.* (1980), *9*, 43-74.

(40) Aota, S. et al. *Nucl. Acids Res.* (1988), *16*, 315-402.

(41) Maroyama, T. et al. *Nucl. Acids Res.* (1986), *14*, 151-197.

(42) Wada, K. et al. *Nucl. Acids Res.* (1991), *19*, 1981-1985.

(43) Kurland, C., *FEBS Letters*, (1991), *285*, 165-169.

(44) Mitsialis, A.; Kafatos F. *Nature*, (1985), *317*, 453-456.

(45) Merriam, J.; Ashburner, M.; Hartl, D; Kafatos, F. *Science*, (1991), *254*, 221-225.

(46) Adang, M.; Miller, L. *J. Virol.*, (1982), *44*, 782-793.

(47) Rohel, D.; Cochran, M.; Faulkner, P. *Virology*, (1983), *124*, 357-365.

(48) Leora Software, (1987). POLO-PC A user's guide to probit or logit analysis, Berkeley, CA.

(49) U.S. Patent No. 5,162,222

(50) Boyce Thompson Institute at Cornell University, Ithaca, NY (Copyright 1990, 1991)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: E. I. DU PONT DE NEMOURS AND COMPANY
(B) STREET: 1007 MARKET STREET
(C) CITY: WILMINGTON
(D) STATE: DELAWARE
(E) COUNTRY: U.S.A.
(F) POSTAL CODE (ZIP): 19898
(G) TELEPHONE: 302-892-8112
(H) TELEFAX: 302-773-0164
(I) TELEX: 6717325

(ii) TITLE OF INVENTION: RECOMBINANT BACULOVIRUS INSECTICIDES

(iii) NUMBER OF SEQUENCES: 13

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: DISKETTE 3.5 INCH
(B) COMPUTER: IBM
(C) OPERATING SYSTEM: MICROSOFT WINDOWS 3.1
(D) SOFTWARE: MICROSOFT WORD FOR WINDOWS 6.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: FLOYD, LINDA AXAMETHY
(B) REGISTRATION NUMBER: 33,692
(C) REFERENCE/DOCKET NUMBER: BA-9063-A

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ACGATGAATT CGGATCCTAT GAAGATCCTC CTTGCTATTG CCCTTATGCT TAGCACCGTG 60

ATGTGGGTGA GCACC 75

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GACGGCTACA TCAAACGCCG CGACGGCTGC AAAGTGGCCT GCCTTATCGG C 51

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AACGAGGGCT GCGACAAAGA GTGCAAAGCC TACGGCGGCA GCTACGGCTA C 51

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGCTGGACCT GGGGCCTCGC ATGCTGGTGC GAGGGCCTCC CCGACGACAA A 51

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ACCTGGAAAA GCGAGACCAA CACCTGCGGC TAAGGATCCT CTAGAGTC 48

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 69 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CACCCACATC ACGGTGCTAA GCATAAGGGC AATAGCAAGG AGGATCTTCA TAGGATCCGA 60

ATTCATCGT 69

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AAGGCAGGCC ACTTTGCAGC CGTCGCGGCG TTTGATGTAG CCGTCGGTGC T 51

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTAGCTGCCG CCGTAGGCTT TGCACTCTTT GTCGCAGCCC TCGTTGCCGA T 51

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTCGGGGAGG CCCTCGCACC AGCATGCGAG GCCCCAGGTC CAGCAGTAGC G 51

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GACTCTAGAG GATCCTTAGC CGCAGGTGTT GGTCTCGCTT TTCCAGGTTT TGTC 54

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 186 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GACGGATATA TAAAAAGACG AGACGGATGC AAGGTTGCAT GCCTGATCGG AAATGAGGGC 60

TGCGATAAAG AATGCAAAGC TTATGGTGGC TCTTATGGAT ATTGTTGGAC CTGGGGACTT 120

GCCTGCTGGT GCGAAGGTCT TCCGGATGAC AAGACATGGA AGAGTGAAAC AAACACATGC 180

GGTTAA 186

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 186 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GACGGCTACA TCAAACGCCG CGACGGCTGC AAAGTGGCCT GCCTTATCGG CAACGAGGGC 60

TGCGACAAAG AGTGCAAGGC CTACGGCGGC AGCTACGGCT ACTGCTGGAC CTGGGGCCTC 120

GCATGCTGGT GCGAGGGCCT CCCCGACGAC AAAACCTGGA AAAGCGAAAC CAACACCTGC 180

GGCTAA 186

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 243 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATGAAGATCC TCCTTGCTAT TGCCCTTATG CTTAGCACCG TGATGTGGGT GAGCACCGAC 60

GGCTACATCA AACGCCGCGA CGGCTGCAAA GTGGCCTGCC TTATCGGCAA CGAGGGCTGC 120

GACAAAGAGT GCAAGGCCTA CGGCGGCAGC TACGGCTACT GCTGGACCTG GGGCCTCGCA 180

TGCTGGTGCG AGGGCCTCCC CGACGACAAA ACCTGGAAAA GCGAAACCAA CACCTGCGGC 240

TAA 243

CLAIMS

What is claimed is:

1. A synthetic gene encoding an insect-selective neurotoxin comprising a coding nucleotide sequence that has been optimized for gene expression based on codon bias of baculoviruses or of cells in which baculoviruses replicate.

2. The synthetic gene of Claim 1 wherein said gene encodes an insect-selective depressant neurotoxin.

3. The synthetic gene of Claim 2 wherein said gene encodes the LqhIT2 insect-selective depressant neurotoxin.

4. The synthetic gene of Claim 1 wherein said optimization is based on codon bias for the coding region of polyhedrin genes of baculoviruses.

5. The synthetic gene of Claim 4 comprising the nucleotide sequence of SEQ ID NO:12.

6. A synthetic gene comprising the synthetic gene of Claim 1 fused in-frame to a nucleotide sequence encoding a suitable signal peptide.

7. The synthetic gene of Claim 6 wherein the suitable signal peptide is derived from bombyxin.

8. The synthetic gene of Claim 7 comprising the nucleotide sequence of SEQ ID NO:13.

9. A chimeric gene comprising the synthetic gene of Claim 6 operably linked to one or more regulatory sequences that direct expression of the coding sequences of the chimeric gene in an insect cell.

10. The chimeric gene of Claim 9 wherein the regulatory sequences comprise baculovirus promoters selected from the group of early promoters, immediately early promoters, late promoters, and very late promoters.

11. A recombinant baculovirus comprising the synthetic gene of Claim 1.

12. A recombinant baculovirus comprising the synthetic gene of Claim 6.

13. A recombinant baculovirus comprising the chimeric gene of Claim 9.

14. The recombinant baculovirus of Claim 13 selected from the group of recombinant baculoviruses designated by ATCC Accession Number ATCC VR-2501 and ATCC Accession Number ATCC VR-2502.

15. An insecticidal composition comprising a recombinant baculovirus according to any one of Claims 11-14 and a suitable carrier therefor.

16. A method for controlling arthropods comprising applying to them or their environment an insecticidally effective amount of the recombinant baculovirus according to any one of Claims 11-14.

17. A method of designing a synthetic, codon-biased gene encoding an insect-selective neurotoxin that is more highly expressed than a non-codon-biased gene

encoding the insect-selective neurotoxin in insect cells infected with baculovirus containing one of the respective genes, the method comprising:

a. determining the coding nucleotide sequence of a non-codon-biased gene encoding the protein to be expressed in a baculovirus-infected insect cell;

b. modifying a portion of the coding nucleotide sequence of the non-codon-biased gene based on codon bias to yield a modified coding nucleotide sequence that contains a greater number of the codons preferred by the baculovirus or by the intended insect host cell than did said coding nucleotide sequence.

18. The method of Claim 17 wherein said modifications are based on codon bias of polyhedrin genes of nuclear polyhedrosis viruses.

Figure 1

```
LqhIT NPV   GACGGCTACA  TCAAACGCCG  CGACGGCTGC  AAAGTGGCCT  40
LqhIT cDNA  GACGGATATA  TAAAAAGACG  AGACGGATGC  AAGGTTGCAT  40

LqhIT NPV   GCCTTATCGG  CAACGAGGGC  TGCGACAAAG  AGTGCAAGGC  80
LqhIT cDNA  GCCTGATCGG  AAATGAGGGC  TGCGATAAAG  AATGCAAAGC  80

LqhIT NPV   CTACGGCGGC  AGCTACGGCT  ACTGCTGGAC  CTGGGGCCTC  120
LqhIT cDNA  TTATGGTGGC  TCTTATGGAT  ATTGTTGGAC  CTGGGGACTT  120

LqhIT NPV   GCATGCTGGT  GCGAGGGCCT  CCCCGACGAC  AAAACCTGGA  160
LqhIT cDNA  GCCTGCTGGT  GCGAAGGTCT  TCCGGATGAC  AAGACATGGA  160

LqhIT NPV   AAAGCGAAAC  CAACACCTGC  GGCTAA                  186
LqhIT cDNA  AGAGTGAAAC  AAACACATGC  GGTTAA                  186
```

Figure 2

Lq1
5'- ACGATGAATT CGGATCCTAT GAAGATCCTC CTTGCTATTG CCCTTATGCT TAGCACCGTG 60
ATGTGGGTGA GCACC - 3' 75

Lq2
5'- GACGGCTACA TCAAACGCCG CGACGGCTGC AAAGTGGCCT GCCTTATCGG C - 3' 51

Lq3
5'- AACGAGGGCT GCGACAAAGA GTGCAAAGCC TACGGCGGCA GCTACGGCTA C - 3' 51

Lq4
5'- TGCTGGACCT GGGGCCTCGC ATGCTGGTGC GAGGGCCTCC CCGACGACAA A - 3' 51

Lq5
5'- ACCTGGAAAA GCGAGACCAA CACCTGCGGC TAAGGATCCT CTAGAGTC - 3' 48

Lq6
5'- CACCCACATC ACGGTGCTAA GCATAAGGGC AATAGCAAGG AGGATCTTCA TAGGATCCGA 60
ATTCATCGT - 3' 69

Lq7
5'- AAGGCAGGCC ACTTTGCAGC CGTCGCGGCG TTTGATGTAG CCGTCGGTGC T - 3' 51

Lq8
5'- GTAGCTGCCG CCGTAGGCTT TGCACTCTTT GTCGCAGCCC TCGTTGCCGA T - 3' 51

Lq9
5'- GTCGGGGAGG CCCTCGCACC AGCATGCGAG GCCCCAGGTC CAGCAGTAGC G - 3' 51

Lq10
5'- GACTCTAGAG GATCCTTAGC CGCAGGTGTT GGTCTCGCTT TTCCAGGTTT TGTC - 3' 54

Figure 3

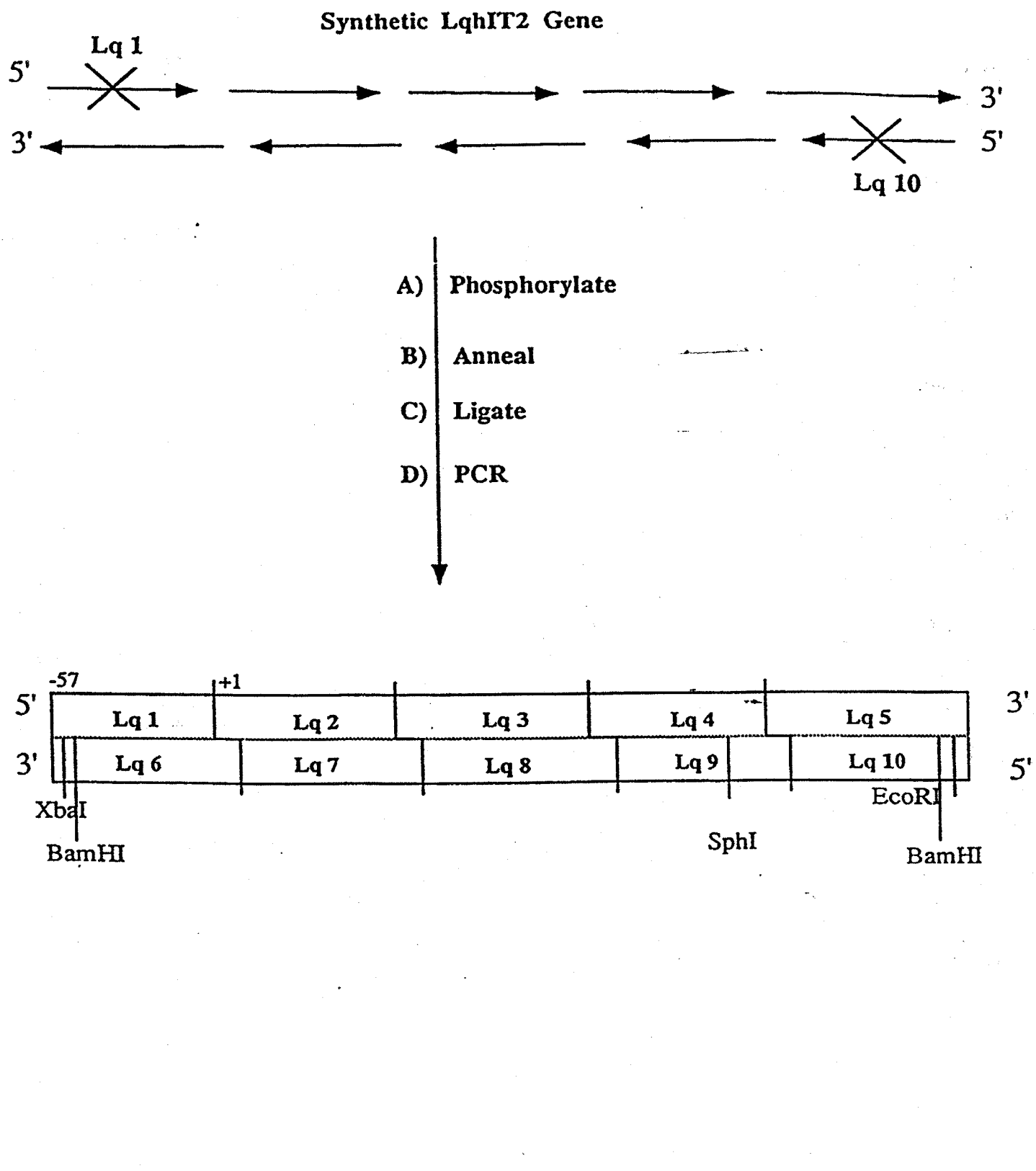

Synthetic LqhIT2 Gene
Lq 1
5'
3'
3'
5'
Lq 10
A) Phosphorylate
B) Anneal
C) Ligate
D) PCR
-57
+1
5'
3'
Lq 1
Lq 2
Lq 3
Lq 4
Lq 5
3'
5'
Lq 6
Lq 7
Lq 8
Lq 9
Lq 10
XbaI
BamHI
SphI
EcoRI
BamHI

Figure 4

LqhIT Seq I.D. # 13 -> 1-phase Translation

DNA sequence 243 b.p. atgaagatcctc ... ACCTGCGGCTAA linear

```
1/1                                                 31/11
atg aag atc ctc ctt gct att gcc ctt atg ctt agc acc gtg atg tgg gtg agc acc GAC
M   K   I   L   L   A   I   A   L   M   L   S   T   V   M   W   V   S   T   D
61/21                                               91/31
GGC TAC ATC AAA CGC CGC GAC GGC TGC AAA GTG GCC TGC CTT ATC GGC AAC GAG GGC TGC
G   Y   I   K   R   R   D   G   C   K   V   A   C   L   I   G   N   E   G   C
121/41                                              151/51
GAC AAA GAG TGC AAG GCC TAC GGC GGC AGC TAC GGC TAC TGC TGG ACC TGG GGC CTC GCA
D   K   E   C   K   A   Y   G   G   S   Y   G   Y   C   W   T   W   G   L   A
181/61                                              211/71
TGC TGG TGC GAG GGC CTC CCC GAC GAC AAA ACC TGG AAA AGC GAA ACC AAC ACC TGC GGC
C   W   C   E   G   L   P   D   D   K   T   W   K   S   E   T   N   T   C   G
241/81
TAA
*
```

Figure 5

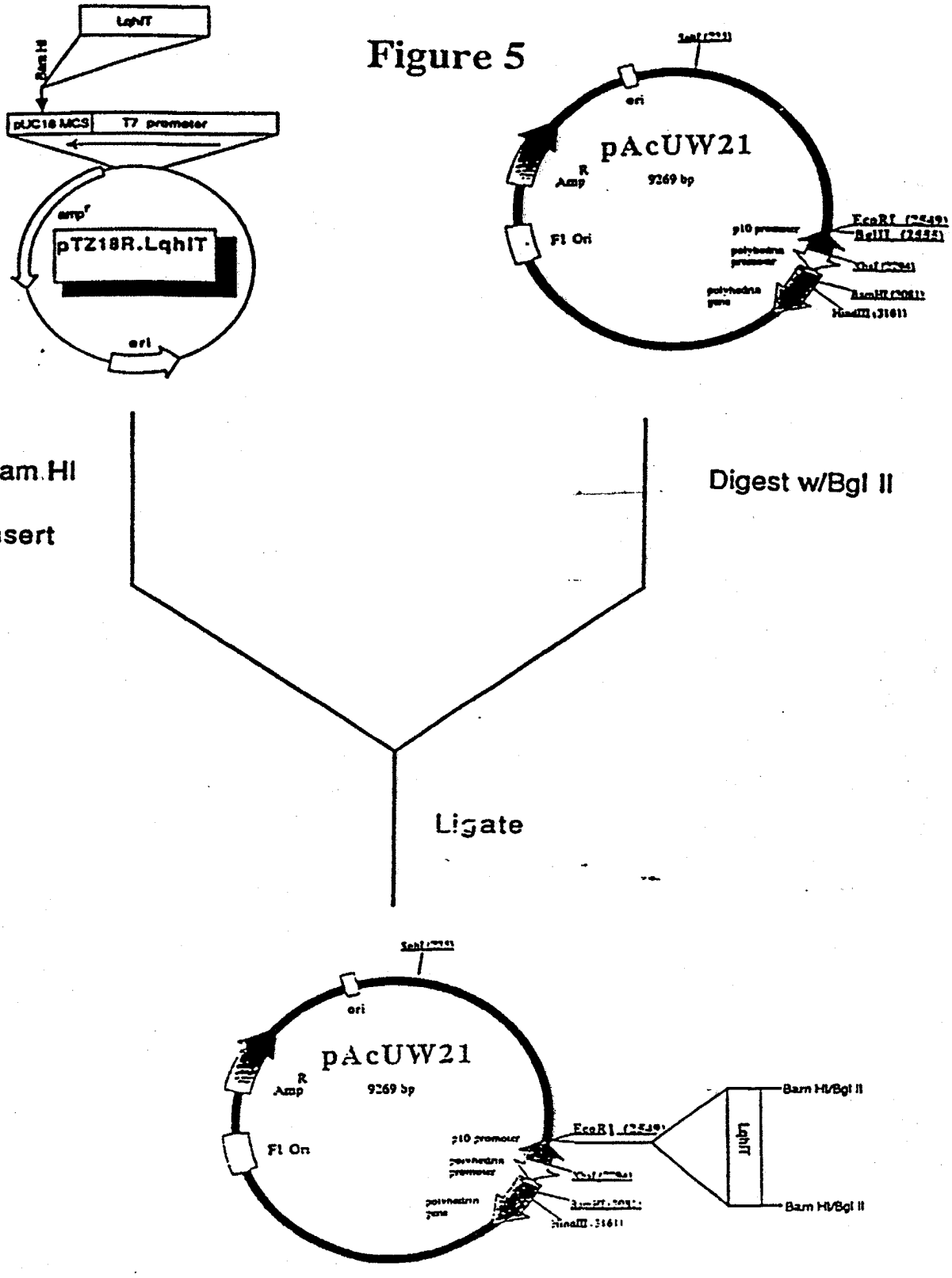

LqhIT
Bam HI
pUC18 MCS
T7 promoter
amp^r
pTZ18R.LqhIT
ori
pAcUW21
9269 bp
Amp R
F1 Ori
p10 promoter
polyhedrin promoter
polyhedrin gene
EcoRI (7549)
BglII (1455)
XbaI (7564)
BamHI (7581)
HindIII (31611)
Digest w/Bam HI
Isolate insert
Digest w/Bgl II
Ligate
Bam HI/Bgl II
Bam HI/Bgl II
LqhIT

Figure 6

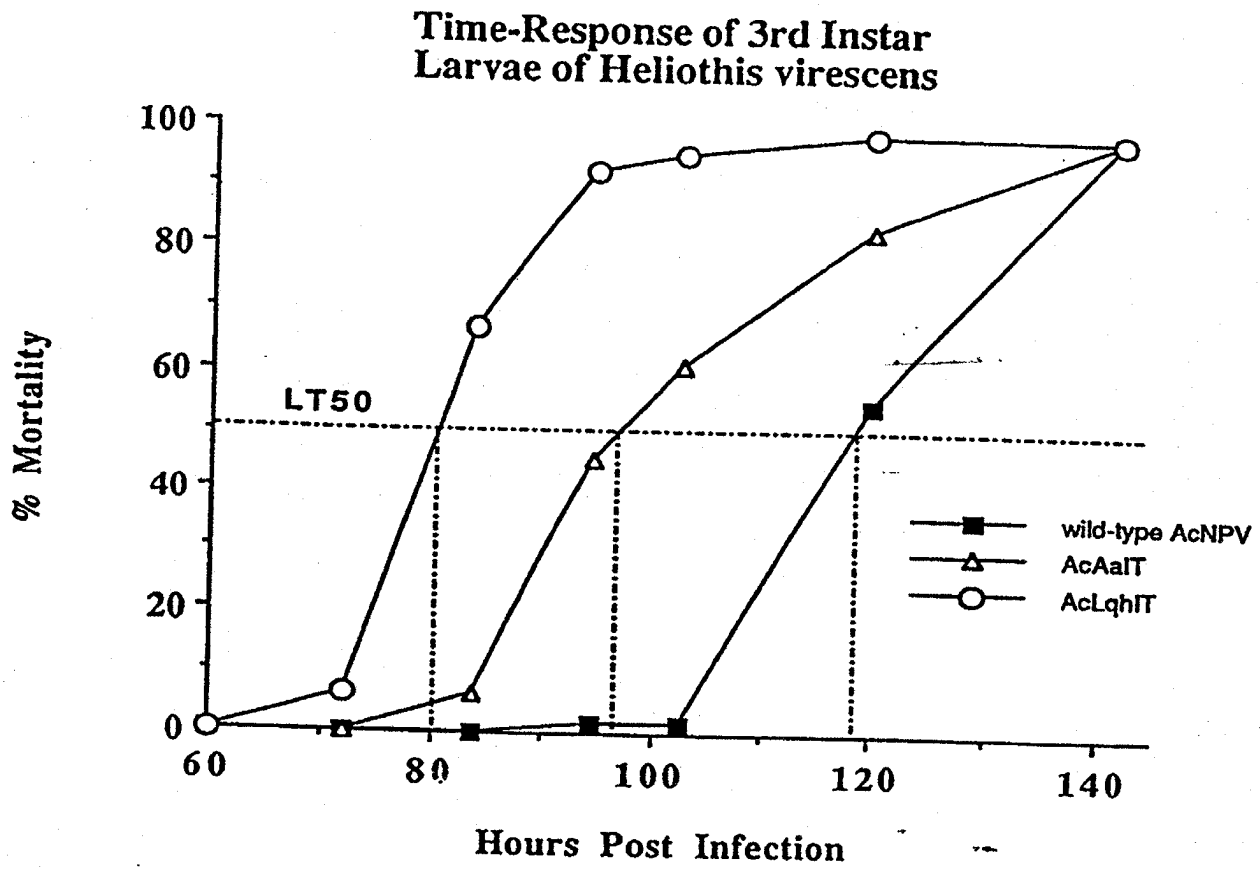
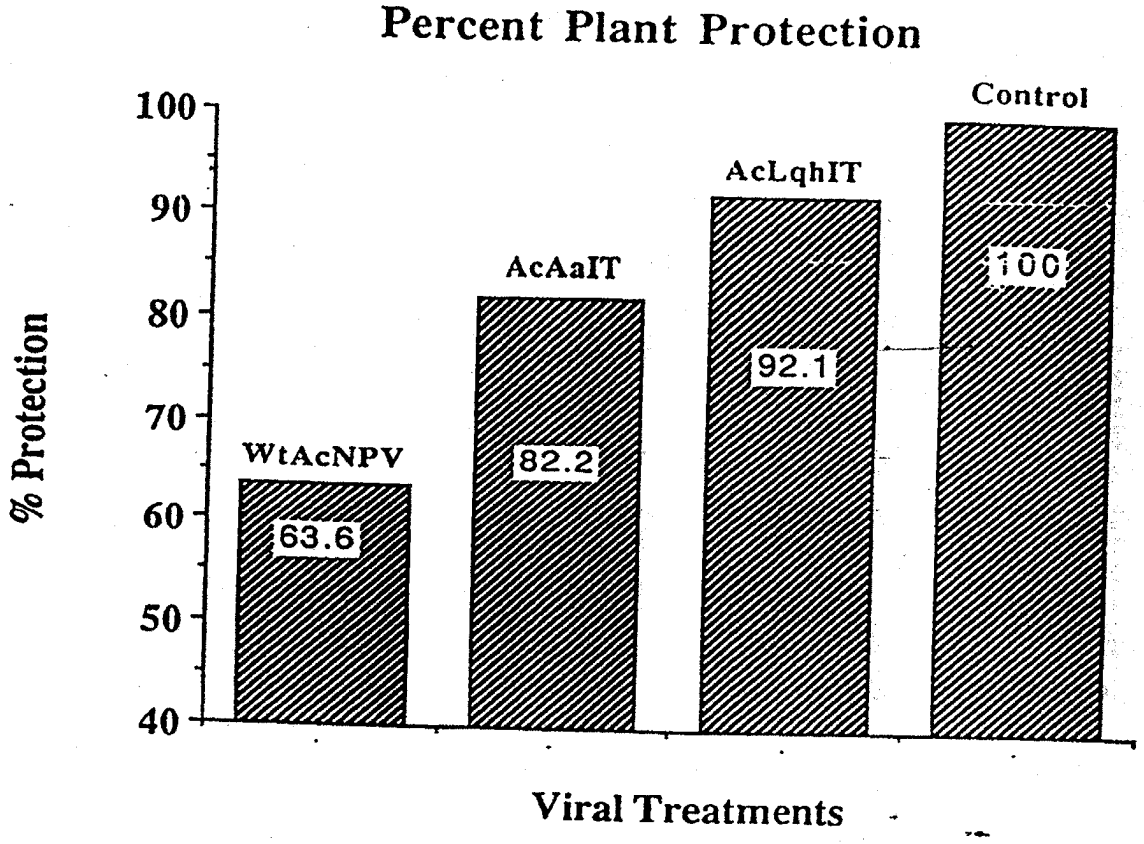

Time-Response of 3rd Instar
Larvae of Heliothis virescens
% Mortality
100
80
60
40
20
0
LT50
60
80
100
120
140
Hours Post Infection
wild-type AcNPV
AcAaIT
AcLqhIT

Figure 7


Percent Plant Protection
100
90
80
70
60
50
40
% Protection
WtAcNPV
63.6
AcAaIT
82.2
AcLqhIT
92.1
Control
100
Viral Treatments

INTERNATIONAL SEARCH REPORT

Internat 1 Application No
PCT/US 96/06988

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/86 A01N63/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP,A,0 621 337 (AMERICAN CYANAMID COMPANY) 26 October 1994 see figure 1; examples 1,2,13	1,2,6, 9-13, 15-17 3,5,7,8, 14
Y	WO,A,92 11363 (CIBA-GEIGY AG) 9 July 1992 see pages 13 and 24-30	3,5,7,8, 14
A	EP,A,0 431 829 (AGRACETUS INC.) 12 June 1991 see column 7, paragraph 2; figure 1	1,2

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 October 1996	18. 10. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cupido, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

...rmation on patent family members

Interna... ...l Application No
PCT/US 96/06988

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0621337	26-10-94	AU-A-	5396794	28-07-94
		CA-A-	2113981	26-07-94
		JP-A-	7313166	05-12-95
		ZA-A-	9400458	01-09-94
WO-A-9211363	09-07-92	AU-A-	9174691	22-07-92
EP-A-0431829	12-06-91	US-A-	5177308	05-01-93
		AU-A-	6706390	06-06-91
		CA-A-	2029451	30-05-91
		JP-A-	3247220	05-11-91

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A01M — A2

(11) International Publication Number: WO 96/36221

(43) International Publication Date: 21 November 1996 (21.11.96)

(21) International Application Number: PCT/US96/06076

(22) International Filing Date: 30 April 1996 (30.04.96)

(30) Priority Data:
08/435,040 8 May 1995 (08.05.95) US

(71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 22nd floor, 300 Lakeside Drive, Oakland, CA 94612-3550 (US).

(72) Inventors: HAMMOCK, Bruce, D.; 3134 Chesapeake Bay Avenue, Davis, CA 95616 (US). HERRMANN, Rafael; 31 Hagefen Street, 27000 Kiryat Bialic (IL). MOSKOWITZ, Haim; Apartment 7, 15 Guatemalla Street, 96704 Jerusalem (IL).

(74) Agents: SIEBERT, J., Suzanne et al.; Majestic, Parsons, Siebert & Hsue, Suite 1450, Four Embarcadero Center, San Francisco, CA 94111-4121 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: INSECT CONTROL WITH MULTIPLE TOXINS

(57) Abstract

A method is provided that accelerates the rate of kill of pests such as from the order *Lepidoptera*. The method comprises treating the pests or their loci with at least two different insect toxins which are expressed from at least one recombinant microbe. Pairs of toxins that do not compete with each other on the same binding site and that differ in their pharmacology have been found to provide synergistic control. Preferred insecticidal microbes are baculoviruses.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

INSECT CONTROL WITH MULTIPLE TOXINS

Field of the Invention

The present invention generally relates to uses of insect toxins in controlling insects, and more particularly to insecticidal recombinant microbes expressing insect-selective toxins in synergistic combinations to magnify insect kill rate.

This invention was made with government support under Grant No. 91-37302-6185, awarded by the United States Department of Agriculture. The U.S. Government has certain rights in the invention.

Background of the Invention

The lepidopteran family Noctuidae includes some of the most destructive agricultural pests, such as the genera *Heliothis*, *Helicoverpa*, *Spodoptera*, and *Trichoplusia*. For example, included in this family are the tobacco budworm (*Heliothis virescens*), the cotton bollworm (*Helicoverpa zea*), the cotton leafworm (*Alabama argillacea*), the spotted cutworm (*Amathes niarum*), the glassy cutworm (*Crymodes devastator*), the bronzed cutworm (*Nephelodes emmedonia*), the fall armyworm (*Laphygma frugiperda*), the beet armyworm (*Spodoptera exigua*), and the variegated cutworm (*Peridroma saucia*).

Resistance of agricultural pests, such as the *Noctuidae* (and others), to pesticides leads to environmental and human health risks. This problem of

insecticide resistance leads to the use of more non-selective and toxic compounds, in order to overcome pest resistance. This creates a destructive and vicious cycle.

Selective natural toxins have been suggested for use in insect control. These toxins include substances which are produced in specialized glandular tissues in the body of a venomous animal. The venom may be introduced into the body of its prey or opponent, such as by the aid of a stinging-piercing apparatus, in order to paralyze and/or kill it, although other means of delivering venom are known. Scorpions, for example, contain in their venom a number of proteins, or neurotoxins, which are toxic and act on the excitable systems. Among the insect specific toxins suggested for use in insect control are toxins from *Bacillus thuringiensis* from the scorpions *Buthus eupeus* and *Androctonus australis*, *Leiurus quinqustriatus hebraeus*, *Leiurus quinqustriatus quinqustriatus*, and from the mite *Pyemotes tritici*.

The venoms derived from scorpions belonging to the Buthinae subfamily have four main groups of polypeptide neurotoxins which modify axonal sodium conductance. One group of scorpion neurotoxins are the α-toxins, which selectively affect mammals through an extreme prolongation of the action potentials due to a slowing or blockage of the sodium channel inactivation (Catterall, *Science*, *223*:653-661 (1984); Rochat et al., *Advances in Cytopharmacology*, pp. 325-334 (1979)). The second group of toxins, the β-toxins, affect sodium channel activation (Couraud and Jover in *Handbook of Natural Toxins* (Tu, A. Ed.) Vol. 2, pp. 659-678 (1984) New York: Marcel Dekker. The third group of neurotoxins are the depressant insect selective toxins which induce a progressively developing flaccid

paralysis of insects by the blockage of action potentials substantially due to the suppression of sodium current (Lester et al., *Biochim. Biophys. Acta*, *701*:370-381 (1982); Zlotkin et al., *Arch. Biochem. Biophys.*, *240*:877-887 (1985)). The fourth group of neurotoxins are the excitatory insect selective toxins which cause an immediate (knock down) spastic paralysis of insects by the induction of repetitive firing in their motor nerves due to an increase of the sodium peak current and the voltage dependent slowing of its inactivation (Walther et al., *J. Insect Physiol.*, *22*:1187-1194 (1976); Pelhate et al., *J. Physiol.*, *30*:318-319 (1981)).

In addition to scorpion and mite toxins, other insect-selective toxins have been identified in venoms from snails, spiders, and a number of other arthropods. [See review by Zlotkin, *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, Vol. 10, Chapter 15, pp. 499-541 (1985).] The venoms of braconid wasps are highly toxic to lepidopterous larvae. The venom of the braconid *Bracon hebetor* causes a flaccid paralysis in lepidopterous larvae by inducing presynaptic interruption of the excitatory glutaminergic transmission at the insect neuromuscular junction (Piek et al., *Comp. Biochem. Physiol.*, *72C*:303-309 (1982)). The venoms of solitary wasps are toxic to a large number of insects and spiders from different orders (Rathmeyer, *Z. Vergl. Physiol.*, *45*:453-462 (1962)). An example of these venoms is the venom of *Philanthus triangulum* which induces in insects a flaccid paralysis substantially due to presynaptic blockage of neuromuscular transmission; this venom affects both excitatory and inhibitory transmission (May et al., *Insect Physiol.*, *25*:285-691 (1979)). The venom of the black widow spider, *Latrodectus mactans*, contains components which are

neurotoxic to insects, but not to mammals, and other components with the opposite selectivity (Fritz et al., *Nature, 238*:486-487 (1980); Ornberg et al., *Toxicon, 14*:329-333 (1976)).

More recently, a toxin designated as LqhαIT, which strongly reassembles α toxins in its primary structure and electrophysiological effects, was isolated from the venom *L. quinquestriatus hebraeus* and was shown to affect mainly insects (Eitan et al., *Biochemistry, 29* (1990), pp. 5941-5947).

The venom of venomous animals is composed of a variety of toxins affecting different target sites in the excitable systems of the prey. On the basis of the data comparing the activity of toxins and their respective crude venom towards lepidopterous larvae it is clear that the potency of the crude venom cannot be explained by the activity of one toxin alone. The higher potency of the crude venom could be related to a cooperativity among different toxins in the venom affecting different target sites on the same ionic channels (Table 3, Trainer et al., *JBC, 268*, 17114-17119 (1993)), different ionic channels on the same excitable cells (Olivera et al., *Science, 249*, 257-263 (1990)), and/or different binding sites on adjacent excitable cells (nerves and/or muscles) (Olivera et al., *Science, 249*, 257-263 (1990)).

The depressant and the excitatory insect-selective toxins do not compete with the α insect toxin for its binding site (Gordon and Zlotkin, *FEBS Lett., 315* (1993), pp. 125-128). In contrast to locust or cockroach neuronal membranes the excitatory toxins do not displace the depressant toxins from their binding sites in neuronal membranes of lepidopterous larvae (Gordon et al., *Biochemisty, 31* (1992), pp. 76-22-7628;

Moskowitz et al., *Insect Biochem. Molec. Biol., 24* (1994), pp. 13-19).

Recently, the nuclear polyhedrosis virus *Autographa californica* (AcNPV), from the family Baculoviridae, has been genetically modified for an increased speed of kill by expressing insect-selective toxins. The introduction of an insect-selective toxin into an insect-pathogenic virus has resulted in a reduction in the killing time of insect hosts, as is described by U.S. Serial No. 08/229,417, filed April 15, 1994, which is a continuation-in-part application of U.S. Serial No. 07/629,603, filed December 19, 1990, having (in part) common assignment herewith.

Tomalski et al., U.S. Patent 5,266,317, issued November 30, 1993, discuss use of recombinant baculoviruses that express an insect-specific paralytic neurotoxin of an insect predacious mite. Barton et al., U.S. Patent 5,177,308, issued January 5, 1993, take a different approach in creating transgenic plants that express a scorpion derived insect-specific toxin and/or a soil dwelling microorganism toxin. In a copending application, of common assignment herewith, Hammock and McCutchen, Serial Number 08/279,956, filed July 5, 1994, discuss insect control with a synergistic combination of recombinant virus and an organic insecticide.

These newly emerging tools using recombinant strategies to control insect pest populations hold promise particularly since the wide-scale presence of pest resistance to organic insecticides, such as pyrethroids, has begun to result in substantial crop losses. In cotton alone, the presence of pyr-R *Heliothis* species has begun to result in millions of lost dollars annually. In fact, in several cases pyrethroid insecticides have completely failed to

control infestations of *Heliothis* larvae in cotton, which has resulted in complete destruction of the crop.

Summary of the Invention

In one aspect of the present invention, a method for controlling a variety of pests is provided through use of genetically engineered, insecticidal microbes. Pests controlled in accordance with the invention are, for example, from the group insects, acarids, and nematodes. Thus, the invention is applicable to the *Lepidoptera* as well as other orders, and to the *Noctuiidae* as well as other families. Such pests are treated (or their loci treated) with a synergistic combination of toxins expressed by one or more recombinant microbes.

For example, the method may use a combination of first recombinant pathogen that expresses a first neurotoxin and a second recombinant pathogen that expresses a second neurotoxin, or may use a single recombinant virus expressing a plurality (such as the first and second) of neurotoxins. The inventive method accelerates the rate of kill of pests by the virus.

Brief Description of Drawing

In the drawing, Figure 1 illustrates the nucleotide sequence of a synthetic gene of LqhIV, SEQ ID NO:1, which one of the preferred toxins for practicing the invention.

Detailed Description of the Preferred Embodiments

The present invention is the use of genetically engineered, insecticidal microbes in combinations to treat pests such as insects. Although recombinant baculoviruses will be used throughout as an illustration of preferred microbes, this invention can

be practiced with a variety of microbes as recombinant delivery systems. Thus the microbes useful in the present invention include DNA and RNA viruses, such as baculoviruses, fungi and bacteria.

On the order of forty nuclear polyhedrosis viruses have been isolated from insect species. (See, for example, *Atlas of Invertebrate Viruses*, Adams and Bonami, editors, CRC Press, Inc., 1991.) Various baculoviruses, including those that infect cotton bollworm, *Helicoverpa zea*, tobacco budworm, *Heliothis virescens*, Douglas fir tussock moth, *Orgia pseudotsugata*, gypsy moth, *Lymantria dispar*, alfalfa looper, *Autographa californica*, European pine fly, *Neodiiprion sertifer*, and codling moth, *Laspeyresia pomonella*, have been registered as pesticides and all such baculoviruses from insect species are suitable for practicing the invention.

Numerous fungi are capable of infecting insects. Introduction of the insect-selective toxin into the genome of such fungi could enhance the potency as pesticides. For example, *Beauvaria bassania* and *Beauvaria brongniartii* have a wide host range and have been suggested as candidates for microbial pesticides (see review by Miller et al., *Science*, *219*:715-721, 1983).

Bacteria (other than *Bacillus thuringiensis*) that have been considered as insect control agents include *Bacillus popilliae*, *B. lentimorbus*, and *B. sphaericus*. Their potential as pesticides can be enhanced by improving their potency through the incorporation of an insect-selective toxin into their genome.

Practice of the invention involves the combined use of two, synergistically acting toxins in controlling insects. These two can be expressed by

means of a single recombinant microbe in which both toxin genes have been introduced, or may be practiced by preparing two recombinant microbes, each of which has been constructed by cloning a gene encoding the respective insect toxins into the genome. The combinations of toxin pairs selected are determined in several ways. As will be described hereinafter (as is described by the screening techniques of Example 6), one preferably selects toxins that act at the same cellular channels (typically sodium channels) but at non-overlapping sites, as will be further described hereinafter.

As earlier mentioned, preferred insecticidal microbes for practicing the invention are baculoviruses. By "baculovirus" is meant any baculovirus of the family *Baculoviridae*, such as a nuclear polyhedrosis virus (NPV). Baculoviruses are a large group of evolutionarily related viruses, which infect only arthropods; indeed, some baculoviruses only infect insects that are pests of commercially important agricultural and forestry crops, while others are known that specifically infect other insect pests. Because baculoviruses infect only arthropods, they pose little or no risk to humans, plants, or the environment.

Of the suitable DNA viruses, in addition to the *Baculoviridae* are the entomopox viruses (EPV), such as *Melolontha melonotha* EPV, *Amsacta moorei* EPV, *Locusta migratoria* EPV, *Melanoplus sanguinipes* EPV, *Schistocerca gregaria* EPV, *Aedes aogypti* EPV, and *Chironomus luridus* EPV. Other suitable DNA viruses are granulosis viruses (GV). Suitable RNA viruses include togaviruses, flaviviruses, picornaviruses, cytoplasmic polyhedrosis viruses (CPV), and the like. The subfamily of double stranded DNA viruses *Eubaculovirinae* includes two genera, NPVs and GVs, which are particularly useful

for biological control because they produce occlusion bodies in their life cycle. Examples of GVs include *Cydia pomonella* GV (coddling moth GV), *Pieris brassicae* GV, *Trichoplusia ni* GV, *Artogeia rapae* GV, and *Plodia interpunctella* GV (Indian meal moth).

Suitable baculoviruses for practicing this invention may be occluded or non-occluded. The nuclear polyhedrosis viruses ("NPV") are one baculovirus sub-group, which are "occluded." That is, a characteristic feature of the NPV group is that many virions are embedded in a crystalline protein matrix referred to as an "occlusion body." Examples of NPVs include *Lymantria dispar* NPV (gypsy moth NPV), *Autographa californica* MNPV, *Anagrapha falcifera* NPV (celery looper NPV), *Spodoptera litturalis* NPV, *Spodoptera frugiperda* NPV, *Heliothis armigera* NPV, *Mamestra brassicae* NPV, *Choristoneura fumiferana* NPV, *Trichoplusia ni* NPV, *Helicoverpa zea* NPV, and *Rachiplusia ou* NPV. For field use occluded viruses often are preferable due to their greater stability since the viral polyhedrin coat provides protection for the enclosed infectious nucleocapsids.

Among illustrative, useful baculoviruses in practicing this invention are those *Anagrapha falcifera*, *Anticarsia gemmatalis*, *Buzura suppressuria*, *Cydia pomonella*, *Helicoverpa zea*, *Heliothis armigera*, *Manestia brassicae*, *Plutella xylostella*, *Spodoptera exigua*, *Spodoptera littoralis*, and *Spodoptera litura*. A particularly useful "NPV" baculovirus for practicing this invention is AcNPV, which is a nuclear polyhedrosis virus from *Autographa californica*. *Autographa californica* is of particular interest because various major pest species within the genera *Spodoptera*, *Trichoplusia*, and *Heliothis* are susceptible to this virus.

The expressed insecticidal toxins are particularly a neurotoxin derived from or similar to an arthropod or other invertebrate toxin, such as a scorpion toxin, a wasp toxin, a snail toxin, a mite toxin, or a spider toxin. A useful scorpion toxin is, for example, AaIT from *Androctonus australis*. Zlotkin et al., *Biochimie*, *53*, 1073-1078 (1971). A useful snail venom is that from the snail *Conus querciones*, which the animal delivers by mouth and some individual toxins of which appear to be selective for arthropods including insects. See, for example, Olivera et al., "Diversity of *Conus* Neuropeptides," *Science*, *249*:257-263 (1990).

Even peptides that normally appear in an insect's developmental life can operate as an insecticidal toxin, and be used in accordance with this invention. For example, the precocious appearance of juvenile hormone esterase ("JHE") will reduce juvenile hormone titers in a host insect, which typically results in irreversible termination of the feeding stage, attempted pupation, and death of the pest insect. The amino acid sequence of JHE is known, and the gene has been cloned. Preferred embodiments of the present invention include recombinant microbes expressing juvenile hormone esterase (JHE) mutations, and exemplary methods for preparing such JHE mutations or deletions, several useful JHE mutations, and recombinant expression vectors for use in controlling insects (having JHE or mutated JHE coding sequences) as are described by WO 94/03588, published February 17, 1994, inventors Hammock et al., incorporated herein by reference.

Two mutants described in the Hammock et al. WO 94/03588 are a double lysine mutant (K29R, K522R) where the normal lysines of JHE at position 29 and position 522 were changed to arginine by site-directed mutagenesis. Another mutant described was where serine

201 was changed to glycine and the mutant designated "S201G." The insecticidal activity of the catalytically deficient S201G mutant of JHE provided similar time for 50% death of test insects to scorpion toxins (when engineered in AcNPV). Thus, the naturally occurring JHE insect protein, which is not normally toxic, can be modified by means such as site-directed mutagenesis (or otherwise) to a toxic agent. In addition to amino acid residue changes, other JHE mutants could be prepared such as by deleting the N-terminal 19 amino acids, which are a signal sequence for the newly made protein to enter the secretory pathway, become ~~glyco~~solated, and exit the cell.

As with JHE, the amino acid sequence of the excitatory toxin from *Androctonus australis* (AaIT) has also been determined, the sequence has been published (Darbon 1982), and the AaIT gene has been cloned and inserted into expression vectors for insect control. (See WO 92/11363, published July 9, 1992, inventors Belagaje et al.) The AaIT toxin exhibits toxicity to insects, while being non-toxic to isopods and mammals.

Yet another suitable toxin for practicing the invention affects insect sodium channels in a manner very similar to the effect of α-toxins on mammalian sodium channels. This neurotoxin was derived from a yellow scorpion *Leuirus quinquestriatus hebraeus*, and is called herein LqhαIT. The identification and purification of this toxin was described in "A Scorpion Venom Neurotoxin Paralytic to Insects that Affects Sodium Current Inactivation: Purification, Primary Structure, and Mode of Action," published by Eitan et al., *Biochemistry*, *29*:5941-5947 (1990).

Two preferred toxins for practicing the invention are novel in isolated and purified form, and will be described more fully hereinafter. Briefly,

these two are designated "LqhIV" and "LqhVI." These two toxins occur in the venom of *L. quinqestriatus hebraeus*, which contains a number of individual toxins in admixture in the native form. The LqhIV toxin is an extremely potent lepidopterous toxin, shows positive cooperativity with other scorpion toxins when injected into the Lepidopterous larvae, and has no or weak mammal toxicity. A synthetic gene for this LqhIV toxin is illustrated by Fig. 1, SEQ ID NO:1.

Thus, the genes for these two preferred toxins can be synthesized (since the peptide sequence sizes are sufficiently small so as to make feasible synthesizing the DNA). Alternatively, the genes can be cloned. The coding sequences may than be cloned into a transfer vector, as will be exemplified further hereinafter.

We have demonstrated aspects of the invention with the synergistic combination of the toxins AaIT and LqhαIT in both blow fly larvae and in *Heliothis* larvae where the insecticidal activity of these insect-selective neurotoxins was increased five to ten fold when used in combination. Other combinations illustrating the invention and experimental details will be more fully discussed hereinafter.

Various other scorpion toxins (e.g. the Buthoid scorpion) can also be used for the synergistic combinations, such as LqqIT2, which is a depressive insect toxin from *Leiurus quinquestriatus quinquestriatus*. The purification method used to obtain this neurotoxin was published by Zlotkin et al., *Archives of Biochem. Biophys.*, *240*:877-887 (1985).

BjIT2 is another depressive insect toxin and is from *Buthotus judaicus*. The purification has been published in Lester et al., *Biochim. Biophys. Acta*, *701*:370-381 (1982). BjIT2 exists in two isoforms which

differ in amino acid sequence at position 15. Form 1 has isoleucine in this position while form 2 has valine.

LqhIT2 is yet another depressive insect toxin from *Leiurus quinquestriatus hebraeus* which was purified using reverse phase HPLC.

Yet other toxins, purified from the venom of the chactoid scorpion, *Scorpio maurus palmatus*, can also be used. For example, SmpIT2, from the chactoid scorpion, *Scorpio maurus palmatus*, is a depressive insect toxin. Its purification is described in Lazarovici et al., *J. Biol. Chem.*, *257*:8397-8404 (1982).

Still other toxins purified ~~from~~ the venom of the chactoid scorpion, *Scorpio maurus palmatus*, are SmpCT2 and SmpCT3, and crustacean toxins, whose purification has been described in Lazarovici, Ph.D. thesis (1980), Hebrew University, Jerusalem, "Studies on the Composition and Action of the Venom of the Scorpion *Scorpio maurus palmatus* (Scorpionidae)."

Table 1 lists some preferred toxins for practicing this invention along with citations to their purification and characterization.

TABLE 1

ILLUSTRATIVE TOXINS	REFERENCES
AaIT	Zlotkin et al., *Biochim*, *53*, 1075-1078 (1971).
$AaIT_1$	Loret et al., *Biochem.*, *29*, 1492-1501 (1990).
$AaIT_2$	Loret et al., *Biochem.*, *29*, 1492-1501 (1990).
$LqqIT_1$	Zlotkin et al., *Arch. f Biochem. & Biophys.*, *240*, 877-887 (1985).
$BjIT_1$	Lester et al., *Biochem. Biophys. Acta*, *701*, 370-387 (1982).

$LqhIT_2$	Zlotkin et al., *Biochem.*, *30*, 4814-4821 (1991).
$LqqIT_2$	Zlotkin et al., *Arch. f Biochem. & Biophys.*, *240*, 877-887 (1985).
$BjIT_2$	Lester et al., *Biochem. Biophys. Acta*, *701*, 370-387 (1982).
$Lqh\alpha IT$	Eitan et al., *Biochem.*, *29*, 5941-5947 (1990).
Ts_{VII}	Bechis et al., *Biochem. Biophys. Res. Comm.*, *122*, 1146-1153 (1984).
Mite toxin	Tomalski et al., *Toxicon*, *27*, 1151-1167 (1989).
α-conotoxins	Gray et al., *JBC*, *256*, 4734-4740 (1981); Gray et al., *Biochem.*, *23*, 2796-2802 (1984).
μ-conotoxins	Cruz et al., *JBC*, *260*, 9280-9288 (1989); Crus et al., *Biochem.*, *28*, 3437-3442 (1989).
chlorotoxin	Debin et al., *Am. J. Physiol.*, *264*, 361-369 (1993).
ω-conotoxins	Olivera et al., *Biochem.*, *23*, 5087-5090 (1984); Rivier et al., *JBC*, *262*, 1194-1198 (1987).
PLTX1	Branton et al., *Soc. Neurosci. Abs.*, *12*, 176 (1986).
PLTX2	Branton et al., *Soc. Neurosci. Abs.*, *12*, 176 (1986).
PLTX3	Branton et al., *Soc. Neurosci. Abs.*, *12*, 176 (1986).
Ag1	Kerr et al., *Soc. Neurosci. Abs.*, *13*, 182 (1987); Sugimori et al., *Soc. Neurosci. Abs.*, *13*, 228 (1987).
Ag2	Kerr et al., *Soc. Neurosci. Abs.*, *13*, 182 (1987); Jackson et al., *Soc. Neurosci. Abs.*, *13*, 1078 (1987).
ω-Agatoxin	Adams et al., *JBC*, *265*, 861-867 (1990).

μ-Agatoxin	Adams et al., *JBC*, *265*, 861-867 (1990).
Hol	Bowers et al., *PNAS*, *84*, 3506-3510 (1987).
α-Laterotoxin	Grasso, in *Neurotoxins in Neurochemistry*, ed. Dolly, 67-79 (1988).
Steatoda toxin	Cavalieri et al., *Toxicon*, *25*, 965-974 (1987).
Bom III	Vargas et al., *Eur. J. Biochem.*, *162*, 589-599 (1987).

---

cDNA libraries for many of ~~the organisms~~ from which the Table 1 illustrative toxins can be purified are available as described by: Zilberberg et al. (1992), *Insect Biochem. Molec. Biol*, *22*(2), 199-203 (*Leiurus quinquestriatus hebraeus*); Gurevitz et al. (1990) *Febs Lett.*, *269*(1), 229-332 (*Buthus judaicus*); Bougis et al. (1989), *JBC*, *264*(32), 19259-19256 (*Androctonus australis*); Martin-Euclaire et al. (1992) *Febs. Lett.*, *302*(3), 220-222 (*Tityus serrulatus*); Woodward et al. (1990) *EMBO J.*, *9*(4), 1015-1020 (*Conus textile*); and Colledge et al. (1992) *Toxicon*, *30*(9), 1111-11116 (*Conus geographus*). For others, one may construct synthetic genes coding for the toxins, in a manner analogous to that exemplified by Example 7.

As earlier mentioned, two toxins suitable for use in practicing the present invention are novel in their isolated and purified form. One of these is designated "LqhIV," and has the amino acid sequence shown as SEQ ID NO:2: GVRDAYIADD KNCVYTCGAN SYCNTECTKN GAESGYCQWF GKYGNACWCI KLPDKVPIRI PGKCR. The SEQ ID NO:2 sixty-five amino acid peptide is further described in Example 5.

Another novel toxin, designed "LqhVI," has the amino acid sequence given by SEQ ID NO:3: GVRDGYIAQP

ENCVYHCFPG SPGCDTLCKG DGASSGHCGF KEGHGLACWC NDLPDKVGII VEGEKCH. This sixty-seven amino acid peptide is also further described by Example 5.

The toxins, such as the preferred toxins listed in Table 1, or as SEQ ID NOS:2 and 3, may be selected to form synergistic combinations most easily by first making experimental combinations of toxins having different pharmacologies. For example, AaIT is an excitatory insect toxin while $LqhIT_2$ is a depressant toxin. By routine binding protocols (see, e.g. Gordon et al., *Biochim. Biophys. Acta*, *778*, 349-358 (1984) for AaIT, $BjIT_1$, and $BjIT_2$ with locust *Locusta migratoria* membrane vesicles), one screens for activity at the same channel but at non-overlapping sites for the particular insect of interest. This is because, as known to the art, there is a variability among various insect neuronal membranes. For example, several recent articles have reported that unlike locust or cockroach neuronal membranes, *Lepidopterous* larvae neuronal membranes can bind the depressant and excitatory insect toxins at the same time.

In the earlier noted example of a synergistic combination of AaIT and LqhαIT, there was twice the synergistic potency for the combination towards blow fly larvae than to *Heliothis* larvae. In contrast, with the combination of AaIT and $LqhIT_2$, there is a synergistic combination (potency of five times) when applied to *Heliothis* larvae, but no increased potency with respect to each toxin by itself when applied to blow fly larvae. These combinations of toxins can be used to enhance selectivity within insect groups.

For producing recombinant microbes, such as baculoviruses, for the purpose of controlling insects, a secretion signal sequence is preferably included. Secretion signal sequences may be derived from proteins

of bacteria, yeast, fungi, or higher eukaryotes, including both animals and plants (for examples, see Watson, *Nucl. Ac. Res.*, *12*:5145-5164 (1984). More preferred are secretion signal sequences from proteins of insect origin, for example those of cecropin B from *Hyalophora cecropia* (van Hofsten et al., *PNAS*, *82*:2240-2243 (1985)), and the eclosion hormone from *Manduca sexta* (Horodyski et al., *PNAS*, *86*:8123-8127 (1989)). Also preferred are the secretion signal sequences naturally associated with scorpion toxins, which can be determined by the analysis of mRNA, cDNA, or genomic DNA. More preferred is the natural secretion signal sequence of AaIT (Bougis et al., *J. Biol. Chem.*, *264*:19259-19265 (1989)).

The toxins of the recombinant microbes may be expressed as functional derivatives of the toxin. A "functional derivative" of the toxin is a compound which possesses a biological activity (either functional or structural) that is substantially similar to a biological activity of the toxin. The term "functional derivative" is intended to include the "fragments," "variants," "analogues," or "chemical derivatives" of a molecule. By a "fragment" of a molecule such as a toxin is meant to refer to any polypeptide subset of the molecule. A "variant" of a molecule such as a toxin is meant to refer to a molecule substantially similar in structure and function to either the entire molecule, or to a fragment thereof. A molecule is said to be "substantially similar" to another molecule if both molecules have substantially similar structures or if both molecules possess a similar biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if the structure of one of the molecules is not found in the other, or if the sequence

of amino acid residues is not identical. An "analog" of a molecule such as the toxin is meant to refer to a molecule substantially similar in function to either the entire molecule or to a fragment thereof. As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule.

Such moieties may improve the molecule's solubility, absorption, biological half-life, etc. Moieties capable of mediating such effects are disclosed in Remington's Pharmaceutical Sciences (1980). Procedures for coupling such moieties to a molecule are well known in the art.

Expression of the toxin (or toxins) will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis. One baculovirus promoter gene is that coding for polyhedrin, since the polyhedrin protein is one of the most highly expressed eucaryotic genes known, although other promoter and hybrid promoter sequences may be used, for example, such as p10.

Recombinant baculoviruses expressing one toxin may be prepared by protocols now known to the art (e.g. Tomalski et al., U.S. Patent 5,266,317, exemplifying neurotoxins from the insect-parasitic mites; McCutchen et al., *Bio/Technology, 9*, 848-852 (1991) and Maeda et al., "Insecticidal Effects of an Insect-Specific Neurotoxin Expressed by a Recombinant Baculovirus," *Virology, 184*, 777-780 (1991), illustrating construction of a recombinant baculovirus expressing AaIT).

Preparation of a single baculovirus capable of expressing two different toxins may by protocols analogous to: Belayev and Roy, *Nucleic Acid Research, 21*:5, 1219-1223 (1993); Wang et al., *Gene, 100*, 131-137

(1991), with appropriate modifications. Example 1 illustrates such an analogous protocol.

EXAMPLE 1

Two insect toxin genes may be cloned into a transfer vector such as PacUW51P2 using standard molecular cloning techniques. This transfer vector is an *Autographa californica* (AcNPV) polyhedron locust-based vector that contains a copy of the AcNPV p10 promoter and SV40 transcription termination signals inserted in tandem, upstream of the polyhedron gene promoter, but in opposite orientation. This facilitates the assertion of one foreign gene coding region at a Bam HI site under the control of the polyhedron promoter, and a second foreign gene coding region at a BglII cloning site, under the control of the p10 promoter. Thus, the resulting recombinant virus expresses two foreign proteins. The recombinant AcNPV thus prepared may be isolated by propagating *Spodoptera frugiperda* cells (Sf21), which are co-transfected by calcium precipitation with the recombinant plasmid. Polyhedron-infected cells can be identified and collected post infection and the recombinant virus plaque purified by screening. Purification of the recombinant virus may be by standard protocols, with the resultant, pure recombinant propagated and stored, such as at 4°C and -80°C. Standard protocols are described, for example, in O'Reilly, Miller and Luckow, *Baculovirus Expression Vectors, A Laboratory Manual*.

EXAMPLE 2

The activities of four different insect toxins towards two different insects and towards mice were determined (since one prefers to use insect toxins that have little or no effect on mammals). These were purified by established methods from the respective crude venoms. Their toxicity towards mice and larvae of blow fly and lepidoptera was determined according to the method of Reed and Muench (1938).

Table 2 shows the activity of the toxins towards insects and mice in terms of ~~fifty~~ percent end points (paralytic or lethal doses PU_{50}, LD_{50} respectively). The PU_{50} values of the toxins to blow fly larvae were in accordance with previously published results (Zlotkin et al., *Biochim*, *53*, 1075-1078 (1971); and Eitan et al., *Biochem.*, *29*, 5941-5947 (1990)). The toxicity of those toxins towards the lepidopterous larvae of *Heliothis virescens* is comparable to their toxicity to larvae of *Spodoptera littoralis*. LqhαIT showed higher toxicity towards mice (Swiss Webster), but the other toxins showed no toxicity to mammals (3 μg/g b.w injected subcutaneously had no effect, in contrast to the LD_{50} of a mammalian toxin AaH_{II} - 0.018 μg/20 g b.w (DeLima et al., 1986). The toxins LqhIV and LqhVI are of considerable interest since LqhIV is the most potent lepidopterous toxin isolated from scorpion venom to date while the toxin LqhVI has weak mammal toxicity.

TABLE 2

Toxins	PU_{50} to *Sarcophaga falculata* larvae (µg/100 mg b.w.)[a]	PU_{50} to *Heliothis virescens* larvae (µg/100 mg b.w.)[b]	LD_{50} to *Swiss Webster* mice (µg/20 g b.w.)[c]
AaIT	0.0025	2.5	>60
$LqhIT_3$	0.050	2.5	>60
$LqhIT_2$	0.025	2.5	>60
LqhαIT	0.0025	2.5	8.0
LqhIV	0.1	0.5	12
LqhVI	0.006	3.0	>60

[a] Three replicates of 25-40 blow fly larvae each were injected with each one of the toxins and the PU_{50} were determined. The PU_{50} of the excitatory toxins AaIT, LqhVI, and $LqhIT_3$ was determined as a contraction paralysis immediately after injection. The PU_{50} of the depressant toxin $LqhIT_2$ was determined as a flaccid paralysis 5 minutes after injection. The PU_{50} of the α insect toxins LqhαIT and LqhIV was determined as a delayed and sustained contraction paralysis 5 minutes after injection.

[b] Three replicates of 25-40 lepidopterous larvae each were injected with each one of the toxins and the PU_{50} was determined as inability to move or turn when inverted on its back 24 hours after injection.

[c] Two replicates of eight mice were injected subcutaneously and the LD_{50} to mice was determined 24 hours after injection.

EXAMPLE 3

Combinations of toxins were injected simultaneously, and toxicity was measured as summarized in Table 3. The toxin combinations included amounts corresponding to 1 PU_{50} unit of each toxin and their dilutions. Pairs of toxins that do not compete with each other on the same binding site and differ in their pharmacology were synergistic. As shown in Table 3, the degree of cooperatively is not only dependent on the toxin combinations but also on the test animal.

TABLE 3

Toxin	PU_{50} to *Sarcophaga falculata* larvae (µg/100 mg b.w.)[a]		PU_{50} to *Heliothis virescens* larvae (µg/100 mg b.w.)[b]		LD_{50} to *Swiss Webster* mice (µg/20 g b.w.)[c]	
	Dose	Change in Potency*	Dose	Change in Potency*	Dose	Change in Potency*
AalT + $LqhIT_2$	0.0025 (AalT) + 0.025 ($LqhIT_2$)	0.5X	0.25 (AalT) + 0.25 ($LqhIT_2$)	5X	60 (AalT) + 60 ($LqhIT_2$)	No effect
AalT + LqhαIT	0.000125 (AalT) + 0.000125 (LqhαIT)	10X	0.25 (AalT) + 0.25 (LqhαIT)	5X	60 (AalT) + 8.0 (LqhαIT)	8.0
$LqhIT_3$ + $LqhIT_2$	Not determined		0.25 ($LqhIT_3$) + 0.25 ($LqhIT_2$)	5X	60 ($LqhIT_3$) + 60 ($LqhIT_2$)	No effect
$LqhIT_3$ + LqhαIT	0.005 ($LqhIT_3$) + 0.00025 (LqhαIT)	5X	0.25 ($LqhIT_3$) + 0.25 (LqhαIT)	5X	60 ($LqhIT_3$) + 8.0 (LqhαIT)	8.0

[a] Three replicates of 25-40 blow fly larvae each were injected with a combination of toxins and the PU_{50} was determined as fast contraction of the larvae within one minute after injection.

[b] Three replicates of 25-40 lepidopterous larvae each were injected with a combination of toxins and the PU_{50} was determined as inability to move or turn when inverted on its back. The PU_{50} was determined 24 hours after injection.

[c] Two replicates of eight mice were injected subcutaneously and the LD_{50} to mice was determined 24 hours after injection.

* The potency was estimated as the amount of toxin protein (a 1:1 ratio of toxins in various dilutions were used) that caused an effect compared to the PU_{50} of each toxin alone.

As illustrated by Table 3, the combinations with greater than one potency were dose-responses greater than potentiation. Thus, these combinations are synergizing the rate of kill and illustrate preferred embodiments of the invention.

EXAMPLE 4

In practicing the invention, pests being controlled are treated (and/or their loci treated) with recombinant baculoviruses expressing such combinations. In this example, the combined application of two viruses expressing two different toxins is shown to reduce the time to kill a host insect when compared to application of each respective virus by itself. Thus, as shown in Table 4, the combined application of the recombinant AcAaIT with the recombinant AcLqhαIT resulted in substantially reduced kill times.

TABLE 4

Recombinant Application	LT_{10}	LT_{50}	LT_{90}
AcLqhαIT (alone)	62	73	87
AcAaIT (alone)	55	68	82
AcAaIT + AcLqhαIT (combined, inventive embodiment)	45	60	80

Lethal times (LT's) were derived based on the response of third instar *H. virescens* larvae to AcAaIT (10000 PIB's), AcLqhαIT (10000 PIB's) and a combined application of AcAaIT (5000 PIB's) and AcLqhαIT (5000 PIB's). Small plugs of diet were placed in individual wells of microtiter plates and inoculated with either of the respective viruses. Third instar larvae of *H. virescens* were then added to the plates and held at 27°C. Mortality was recorded at 5-10 intervals. LT's were analyzed with a Probit analysis program.

Thus, the data of Table 4 is a study of speed of kill expressed as lethal times (LTs) and analogous approaches can be used to determine lethal doses, which are likely to be of major economic importance. Taking,

for example, the lethal time at which 50% of the larvae were dead, one sees that the combination of toxins in practicing the inventive method provided approximately a 12% to 18% reduction in the time required to kill the host larvae with respect to applications of individual recombinants. When one considers that treatment with the recombinant AcAaIT represents an approximate 40% reduction in the time required to kill host larvae when compared to wild-type AcNPV, one sees a substantial decrease in insect feeding damage and significantly less damaged plants result from practice of the invention. Further, larvae infected with recombinant microbes typically start showing symptoms of paralysis and stop feeding a number of hours prior to death, which further increases the practical insecticidal effects of the inventive method.

EXAMPLE 5

Purification of LqhIV and LqhVI

Venom of the scorpion *L. quinqestriatus hebraeus* was obtained from Sigma (USA).

Lyophilized *L. quinqestriatus hebraeus* venom (50 mg) was suspended and homogenized in 2 ml of 10 mM Ammonium acetate pH=6.4. The insoluble material removed by centrifugation at 27000g for 20 min. The supernatant was collected and the pellet was resuspended in additional 2 ml of 10 mM ammonium acetate pH=6.4, homogenized and centrifuged again. This extraction was done 4 times to maximize the yield of protein extracted from the venom. The supernatant from all the centrifugations was pooled, loaded on a cation exchanger column (10 ml of CM-52) and eluted with a linear gradient of 0.01-0.5 M ammonium acetate pH=6.4 in a flow

rate of 10 ml/hr. Absorbance was monitored at 280 nm and peaks collected accordingly. The fraction CM-III and CM-VI from the cation exchanger chromatography were further purified on RP-HPLC on Vydac C4 Column. LqhIV was purified from CM-VI as follows: buffer A was 5% ACN with 0.1% TFA and buffer B was 95% ACN with 0.1% TFA. The column was equilibrated in buffer A and eluted with a linear gradient of 0-60% B in 70 min, the flow rate was 0.6 ml/min. Absorbance was monitored at 214nm and peaks collected accordingly LqhVI was purified from CM-III as follows: buffer A was 5% ACN with 0.1% HFBA and buffer B was 95% ACN with 0.1% HFBA. The column was equilibrated in buffer A and eluted with a linear gradient of 0-90% B in 105 min, the flow rate was 0.6 ml/min. Absorbance was monitored at 214 nm and peaks collected accordingly. The eluted fractions were collected and tested for activity (Table 2) and purity.

Purity of toxins

The homogeneity and purity of LqhIV and LqhVI was tested by Free Solution Capillary Electrophoresis (Applied Biosystems Model 270A). The capillary was equilibrated with 20 mM Sodium citrate pH=2.9 and the samples (0.02 mg/ml protein) were loaded using vacuum for two seconds. The running buffer was 20 mM sodium citrate pH=2.9, the electric force was 20KV.

Sequence determination

20 μg of each toxin were reduced and carboxymethylated using established method (Fernandez et al., "Techniques in Protein Chemistry," Vol. 5 page 215). The N-terminal sequence was determined using HP sequence analyzer by automated Edman degradation. Reduced and carboxymethylated LqhIV was digested using Endoproteinase Asp-N and peptides were generated.

Separation of the digested peptides was done on microbore HPLC (Ultrafast Microprotein analyzer- Michrom BioResources Inc) using polymeric column. Buffer A was 5% ACN with 0.1% TFA and buffer B was 95% ACN with 0.1% TFA. The column was equilibrated in buffer A and eluted with a linear gradient of 0-50% B in 50 min, the flow rate was 0.05 ml/min. Absorbance was monitored at 214 nm and peaks collected accordingly. Peptide P2 was sequenced in order to determine the full amino acid sequence of the toxin.

EXAMPLE 6

Binding Protocol

Preparation of Insect Neuronal Membranes

All dissections and preparations of insect neuronal tissues are performed in a cold buffer of the following composition: 0.25 M mannitol, 10 mM EDTA pH=7.4, 5 mM HEPES (adjusted to pH 7.4 with Tris), 50 μg/ml phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 1 mM iodoacetamide and 1 mM 1,10-phenantroline. Insect nervous tissues are dissected and homogenized in ice cold buffer, the debris is removed by centrifugation at 1,000 g for 10 min. The supernatant is centrifuged at 27,000g for 45 min and the membranes are collected (P_2). The P_2 is suspended in the buffer and adjusted to 10% Ficoll (in the buffer) and centrifuged at 10,000 g for 75 min. The resulting floating pellicle representing the enriched synaptosomal fraction is collected. Following treatment by hypotonic medium (5 nM Tris-HCl pH=7.4, 1 mM EDTA, 50 μg/ml phenylmethylsulfonyl fluoride, 1μM pepstatin A, 1 mM iodoacetamide and 1 mM 1,10-phenantroline) membrane vesicles are formed. The membrane vesicles are collected in a small volume of

dissection buffer after centrifugation 27,000 g for 45 min and stored at -80°C until use.

Radioiodination of Toxins

The toxins are iodinated by iodogen (Pierce Chemical Co., Rockville, MD) using 0.5 mCi of carrier-free $Na^{125}I$ (~0.3 nmol) (Amersham) and 5 mg (~0.7 nmol) of toxin. The monoiodotoxin is purified on HPLC using a Beckman Ultrapore C3 RPSC column (4.6x75 mm) fractions are eluted at gradient of 10-80% solvent B (solvent A = 0.1% TFA, solvent B = 50% ACN, 50% 2-propanol and 0.1% TFA) at flow rate of 0.5 ml/min. The monoiodotoxin is eluted as the first peak of radioactive protein (about 30% solvent B) following the peak of the native toxins (about 28% solvent B). The concentration of the radiolabeled toxin is estimated according to the specific radioactivity of ^{125}I and correspond to 2424 dpm/fmol monoiodotoxin.

Binding Assays

Competitive binding assays are performed at equilibrium conditions using increasing concentrations of an unlabeled toxin in the presence of a constant concentration of a labeled toxin. Analysis of all binding assays is performed using the iterative computer program LIGAND (P.J. Munson and D. Rodbard, modified by G.A. McPherson 1985). Insect membrane vesicles are suspended in binding medium containing 0.13 M choline chloride, 1 mM EDTA pH=7.4, 20 mM HEPES/Tris pH=7.4 and 5 mg/ml BSA. Following 1 hour incubation with the toxins, the reaction mixture is diluted with 2 ml ice-cold wash buffer (150 mM choline chloride, 5 mM HEPES/Tris pH=7.4, 1 mM EDTA pH=7.4 and 5 mg/ml BSA) and filtered over GF/F filters (Whatman, U.K.) under vacuum, followed by two more washes of the filters with 2 ml of

wash buffer each time. Non-specific toxin binding is determined in the presence of 1 μM unlabeled toxin.

EXAMPLE 7

Construction of the Synthetic Gene (Fig. 1, SEQ ID NO:1)

The protein sequence of a toxin was converted into a nucleotide sequence using the preferred codon usage of a baculovirus. The toxin gene along with the nucleotide sequence of a leader sequence (bombyxin, native leader or other) and the appropriate restriction enzyme sites were used to design and synthesize 5 complementary pairs of oligonucleotides. The oligonucleotides were phosphorilated, annealed, ligated and amplified by PCR using the outside oligonucleotides as primers. The PCR product is blunt end ligated into a PCRscript plasmid and the correct sequence was confirmed by sequencing. A BamHI restriction fragment was rescued from this plasmid cloned into a baculovirus transfer vector under a baculovirus promoter (P10, polyhedrin, Basic, IE1 etc.). A plasmid containing the correct sequence of the gene and leader sequence was confirmed by sequencing. Using the resulting transfer vector and the standard procedures a recombinant virus expressing the toxin was constructed.

Construction of a Virus Expressing AaIT and LqhIV

The leader sequence of bombyxin and the gene coding for the toxin LqhIV were designed and synthesized as described above. The correct sequence was confirmed and the gene was cloned into a double expression transfer vector already containing the AaIT gene. The transfer vector pAcUW51P2 is a polyhedrin positive vector with two cloning sites, a BglII site and the LqhIV gene with the bombyxin leader were cloned into

the BamHI site. Sf21 cells were cotransfected with the resulting transfer vector and infectious virus particles using the lipofectin procedure. Recombinant viruses were selected as a polyhedrin positive phenotype in a standard plaque assay. Sf21 cells were inoculated with the recombinant virus AcAaLq according to standard procedures. Protein extracts from virus infected cells were separated on 15% SDS-PAGE gels and then electroeluted to nitrocellulose membranes. The membranes were probed with AaIT and LqhIV antibodies, bound antibodies were detected using rabbit IgG HRP conjugates.

In conclusion, genetically engineered, insecticidal microbes are produced in accordance with the invention and then used to control a variety of pests. In doing so, one may use a single recombinant virus expressing a plurality of neurotoxins. The combination of toxins are determined by selecting toxins that act at the same cellular channels (typically sodium channels) but at non-overlapping sites. Alternatively, one may use two (or more) recombinant insecticidal microbes where each expresses a different toxin. Again, the several expressed toxins are selected as already described. These combinations of expressed toxins accelerate the rate of kill of pests by the virus or viruses beyond simply an "additive" function. For example, the lethality of toxins AaIT and LqhαIT in both blowfly larvae and in *Heliothis* larvae was increased 5-10 fold when used in combination. Further, the combinations of toxins can be used to enhance selectivity within insect groups.

Conventional application means of the recombinant microbes (spraying, atomizing, dusting, scattering, or pouring) may be used from formulations

such as powders, dusts, granulates, as well as encapsulations such as in polymer substances. Compositions will typically include inert carriers such as clay, lactose, defatted soy bean powder, and the like to assist in applications, in order to apply the recombinant microbes expressing synergistic combinations of insecticidal toxins.

It is to be understood that while the invention has been described above in conjunction with preferred specific embodiments, the description and examples are intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.

It Is Claimed:

1. A method for controlling pests from the group insects, acarids, and nematodes comprising:

treating said pests or their loci with at least two different insect toxins, the source of the toxins being at least one recombinant microbe or being a plurality of recombinant microbes, the toxins having non-overlapping binding sites at an insect cellular membrane channel.

2. The method as in claim 1 wherein the source of the toxins are recombinant insect viruses.

3. The method as in claim 1 wherein the source of recombinant insect viruses are baculoviruses.

4. The method as in claim 3 wherein the baculoviruses are nuclear polyhedrosis viruses.

5. The method as in claim 3 wherein the baculoviruses are from *Autographa californica, Anagrapha falcifera, Anticarsia gemmatalis, Buzura suppressuria, Cydia pomonella, Helicoverpa zea, Heliothis arrigera, Mariestia brassicae, Plutella xylostella, Spodoptera exigua, Spodoptera littoralis*, or *Spodoptera litura*.

6. The method as in claim 1 wherein the toxins are combinations of AaIT, $LqhIT_2$, LqhαIT, and $LqhIT_3$ neurotoxins.

7. The method as in claim 1 wherein the toxins include a JHE mutant.

8. The method as in claim 6 or 7 wherein the pest is *Heliothis virescens* or blow fly.

9. A substantially pure insect toxin, useful in insect control, having the amino acid sequence SEQ. ID NO:1 or SEQ. ID NO:2.

10. A recombinant microbe which, in insect cells infected therewith, expresses at least two foreign proteins toxic to the insect cells, or functional derivatives thereof, the microbe genome provided with a secretion signal sequence.

11. The recombinant microbe as in claim 10 wherein the microbe is a nuclear polyhedrosis virus.

12. The recombinant microbe as in claim 10 or 11 wherein at least one of the foreign proteins is a scorpion toxin.

13. The recombinant microbe as in claim 10 wherein the foreign proteins are derived from genetic sequence codings of scorpion, wasp, snail, mite, or spider venoms.

14. The microbe as in claim 10, 12, or 13 wherein the baculovirus is *Autographa californica* nuclear polyhedrosis virus.

15. An insect control composition comprising:

a first and a second recombinant baculovirus, the first and second baculoviruses respectively expressing a first and a second toxin, the first and second toxins having non-overlapping binding sites at an insect cellular membrane channel.

16. An insect control composition comprising:

a recombinant baculovirus which, in insect cells infected therewith, expresses a plurality of insecticidal toxins.

AGA TCT GGA TCC ATG AAG ATC CTC CTT GCT ATT GCC CTT ATG CTT AGC ACC GTG
TCT AGA CCT AGG TAC TTC TAG GAG GAA CGA TAA CGG GAA TAC GAA TCG TGG CAC

ATG TGG GTG AGC ACC GGC GTG CGC GAC GCC TAC ATC GCC GAC GAC AAG AAC TGC
TAC ACC CAC TCG TGG CCG CAC GCG CTG CGG ATG TAG CGG CTG CTG TTC TTG ACG

GTG TAC ACC TGC GGC GCC AAC TCT TAC TGC AAC ACC GAC TGC ACC AAG AAC GGC
CAC ATG TGG ACG CCG CGG TTG AGA ATG ACG TTG TGG CTC ACG TGG TTC TTG CCG

GCC GAC TCT GGC TAC TGC CAA TGG TTC GGC AAA TAC GGC AAC GCA TGC TGG TGC
CGG CTC AGA CCG ATG ACG GTT ACC AAG CCG TTT ATG CCG TTG CGT ACG ACC ACG

ATC AAA CTT CCC GAC AAA GTG CCC ATC CGC ATT CCC GGC AAA TGC CGC TAA GGA
TAG TTT GAA GGG CTG TTT CAC GGG TAG GCG TAA GGG CCG TTT ACG GCG ATT CCT

TCC AGA TCT GAG CTC
AGG TCT AGA CTC GAG

Fig. 1

Pergamon

PII: S0041-0101(98)00080-4

Toxicon Vol. 36, No. 8, pp. 1141–1154, 1998

Printed in Great Britain
0041-0101/98 $19.00 + 0.00

NEW TOXINS ACTING ON SODIUM CHANNELS FROM THE SCORPION *LEIURUS QUINQUESTRIATUS HEBRAEUS* SUGGEST A CLUE TO MAMMALIAN VS INSECT SELECTIVITY

PIERRE SAUTIÈRE,[1] SANDRINE CESTÈLE,[2] CHARLES KOPEYAN,[3] ARLETTE MARTINAGE,[4] HERVÉ DROBECQ,[1] YVON DOLJANSKY[5] and DALIA GORDON[1,6]*

[1]Laboratoire de Chimie des Biomolécules, URA 1309 CNRS, Institut Pasteur de Lille, 1 Rue du Professeur Calmette, F-59019 Lille, France, [2]Laboratoire de Biochimie, Ingénierie des Protéines, CNRS URA 1455, I.F.R. Jean Roche, Faculté de Médecine Nord, Bd Pierre Dramard, 13916 Marseille, France, [3]Latoxan, A.P. 05150 Rosans, France; and [4]CEA, Département d'Ingénierie et d'Etudes des Protéines, Bat. 152 - C.E. Saclay, 91191 Gif-sur-Yvette Cedex, France

(*Received* 27 *March* 1997; *accepted* 22 *January* 1998)

P. Sautière, S. Cestèle, C. Kopeyan, A. Martinage, H. Drobecq, Y. Doljansky and D. Gordon. New toxins acting on sodium channels from the scorpion *Leiurus quinquestriatus hebraeus* suggest a clue to mammalian vs insect selectivity. *Toxicon* 36, 1141–1154, 1998.—Two new toxins were purified from *Leiurus quinquestriatus hebraeus* (Lqh) scorpion venom, Lqh II and Lqh III. Lqh II sequence reveals only two substitutions, as compared to AaH II, the most active scorpion α-toxin on mammals from *Androctonus australis* Hector. Lqh III shares 80% sequence identity with the α-like toxin Bom III, from *Buthus occitanus mardochei*. Using bioassays on mice and cockroach coupled with competitive binding studies with ^{125}I-labeled scorpion α-toxins on rat brain and cockroach synaptosomes, the animal selectivity was examined. Lqh II has comparable activity to mammals as AaH II, but reveals significantly higher activity to insects attributed to its C-terminal substitution, and competes at low concentration for binding on both mammalian and cockroach sodium channels. Lqh II thus binds to receptor site 3 on sodium channels. Lqh III is active on both insects and mammals but competes for binding only on cockroach. The latter indicates that Lqh III binds to a distinct receptor site. Thus, Lqh II and Lqh III represent two different scorpion toxin groups, the α- and α-like toxins, respectively, according to the structural and pharmacological criteria. These new toxins may serve as a lead for clarification of the structural basis for insect vs mammal selectivity of scorpion toxins. © 1998 Elsevier Science Ltd. All rights reserved

*Author to whom correspondence should be addressed.

INTRODUCTION

The principle toxic compounds in scorpion venoms are toxic polypeptides that interfere with the sodium conductance in mammalian excitable tissues. The sodium channel neurotoxins from scorpion venom defined a family of homologous polypeptides, composed of a single chain of 63–70 amino acid residues cross-linked by four disulfide bridges (Miranda *et al.*, 1970; Martin-Eauclaire and Couraud, 1995; Gordon *et al.*, in press) and were classified into several structural groups on the basis of primary structure (Rochat *et al.*, 1979; Dufton and Rochat, 1984; Possani, 1984; Watt and Simard, 1984) and immunological (Delori *et al.*, 1981) criteria. The four first groups (I IV) contain the scorpion α-toxins and, the newly defined group of α-like toxins (Gordon *et al.*, 1996), active on vertebrates (Martin-Eauclaire and Couraud, 1995; Gordon *et al.*, in press). The phylogenetic specificity of these toxins vary considerably (Zlotkin *et al.*, 1978). Thus, toxins specifically active on mammals (Miranda *et al.*, 1970), insects or crustaceans have been already described (Zlotkin, 1986). All these different toxins affect sodium conductance in various excitable tissues, and serve as important pharmacological tools for the study of excitability and sodium channel structure and function.

At least seven neurotoxin receptor sites have been identified by direct radiolabeled toxin binding and competition binding studies on the mammalian sodium channels, and additional as yet unidentified receptor sites have been noticed (Gordon, 1997a; Trainer *et al.*, 1997). Although the identification and characterization of the distinct receptor sites have been predominantly performed using vertebrate excitable preparations (Catterall, 1980, 1986; Strichartz *et al.*, 1987), insect neuronal membranes have been shown to possess similar receptor sites. Insect sodium channels were shown to resemble their vertebrate counterparts by their primary structure (Loughney *et al.*, 1989), topological organization (Gordon *et al.*, 1992; Moskowitz *et al.*, 1994), and basic biochemical (Gordon *et al.*, 1988, 1990, 1992, 1993; Moskowitz *et al.*, 1991, 1994) and pharmacological (Pelhate and Sattelle, 1982; Cestele *et al.*, 1995) properties. On the other hand, a possible uniqueness of the insect sodium channels was suggested by the description of two groups of scorpion toxins that modify sodium conductance exclusively in insect neuronal preparations, the excitatory and depressant insect selective toxins (Pelhate and Zlotkin, 1982; Zlotkin *et al.*, 1985, 1991). These toxins bind selectively to insect sodium channels at two distinct receptor sites (Gordon *et al.*, 1992; Moskowitz *et al.*, 1994) and, therefore, indicate the existence of unique features in the structure of insect channels, as compared to their mammalian counterparts (Gordon *et al.*, 1984, 1992, 1993, 1996). Thus, a comparative study of mammalian and insect neurotoxin receptor sites on the respective sodium channels may elucidate the structural features involved in the binding and activity of the various neurotoxins, and may contribute to the clarification of structure-function relationship in sodium channels.

Receptor sites for peptide neurotoxins that inhibit sodium current inactivation in neurons (the classical effect induced by α-scorpion and sea anemone toxins) are of particular interest for the study of the dynamics of channel gating, since neurotoxin binding at these extracellular regions can affect the inactivation process at intramembranal segments of the channel (Catterall, 1992; Gordon, 1997a,b). The most studied neurotoxins that induce inhibition of sodium current inactivation are the scorpion α-toxins and sea anemone toxins, that were shown to bind to overlapping region comprising receptor site 3 on rat brain (Catterall and Beress, 1978; Couraud *et al.*, 1978; Rogers *et al.*, 1996) and insect (Gordon and Zlotkin, 1993; Gordon *et al.*, 1996) sodium channels. Several scorpion α-toxins have been identified by their high toxicity to mammals and by a high

homology in their amino acid sequence (Reviewed by Martin-Eauclaire and Couraud, 1995). Two scorpion α-toxins highly active on insects have been recently pharmacologically characterized, LqhαIT and Lqq III (Eitan *et al.*, 1990; Gordon and Zlotkin, 1993; Gordon *et al.*, 1996; Cestele *et al.*, 1997). These two highly homologous α-toxins (see Fig. 3) were shown by competitive binding studies to be selective probes for receptor site 3 on insect sodium channels (Gordon and Zlotkin, 1993; Gordon *et al.*, 1996; Cestele *et al.*, 1997). Recently, we have described a new group of scorpion toxins that affect sodium current inactivation and is active on both mammals and insects, the so-called scorpion α-like toxins (Gordon *et al.*, 1996). Our study suggested that scorpion toxins affecting inactivation of sodium channels may be divided to several different groups according to their mammal vs insect activities and their binding properties, each possessing a putative distinct receptor site on sodium channels (Gordon *et al.*, in press).

In the present report we describe the purification and pharmacological characterization of two new scorpion toxins, designated Lqh II and Lqh III, from the venom of the Israeli yellow scorpion *Leiurus quinquestriatus hebraeus*, that represent by their primary structure the scorpion α-toxins and the α-like scorpion toxin groups, respectively. We have used AaH II, the scorpion α-toxin most active on vertebrates, that reveals the highest affinity to rat brain synaptosomes (Jover *et al.*, 1978), and LqhαIT, the scorpion α-toxin that reveals high activity on insects (Eitan *et al.*, 1990; Gordon *et al.*, 1996), as specific probes for receptor site 3 in rat brain and insect sodium channels, respectively. Interestingly, Lqh II reveals an unusual homology to AaH II, having only two amino acid substitutions at its N- and C-termini. Moreover, this toxin has comparable activity to mammals as AaH II, but reveals significantly higher activity to insects, attributed to the C-terminal substitution. The second toxin, Lqh III, reveals sequence similarity to the previously described α-like scorpion toxin group (Bom III and Bom IV, Gordon *et al.*, 1996, and unpublished) and is active on both insects and mammals.

MATERIALS AND METHODS

Toxins and fractions

AaH II was purified according to Miranda *et al.* (1970) and was kindly provided by Prof. Herve Rochat, Laboratoire de Biochimie, Ingénierie des Protéines, CNRS URA 1455, Faculté de Médecine Nord, Marseille, France. Recombinant LqhαIT was expressed and prepared according to Zilberberg *et al.* (1996) and was a generous gift of Prof. Michael Gurevitz and Noam Zilberberg, Department of Botany, Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel. Ion-exchange fractions from the venom of the scorpion *Leiurus quinquestriatus hebraeus* obtained according to the procedure described by Marshall *et al.* (1994) were provided by Latoxan (A.P. 05150 Rosans, France). The new toxins, Lqh II and Lqh III purified from these fractions are designated as LTx-001 and LTx-002, respectively, in the Latoxan catalogue. All reagents and solvents were of the highest purity available.

Enzymes

Trypsin (E.C.3.4.21.4) treated with tosylphenylalanyl-chloromethane was from Sigma. Chymotrypsin (E.C.3.4.21.1.) treated with tosyllysyl-chloromethane was obtained from Merck. Endoproteinase Glu-C (E.C.3.4.21.19) and carboxypeptidase P (E.C.3.4.17.16) sequencing grade were purchased from Boehringer (Mannheim, Germany).

Purification procedure

Ion-exchange fractions 3 and 11 were submitted to preparative RP-HPLC on a Nucleosil column C18 (5 μM, 100 Å pore size, 500 × 10 mm, Machercy Nagel) eluted at a flow-rate of 2.5 ml min^{-1} with a gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA). The purity of polypeptides after preparative RP-HPLC was assessed by capillary electrophoresis (270A-HT Capillary Electrophoresis System, Applied Biosystems) in 20 mM sodium citrate buffer pH 2.5, at 30 kV and 30°C for 10 min, using a 50 cm-capillary, 50 μm in diameter. Samples were injected for 1 s under a 5 inch vacuum. UV detection was performed at 200 nm.

Mass spectrometry

The molecular masses of the native polypeptides and of their fragments generated from enzymatic cleavages were determined by ion-spray mass spectrometry, on freeze-dried samples dissolved in 20% acetonitrile and 0.1% formic acid in water, at a concentration of 20 pmol μl^{-1}. Ion-spray mass spectra were recorded on a simple-quadrupole mass spectrometer API I (Perkin-Elmer Selex) equipped with an ion-spray (nebulizer-assisted electrospray) source (Sciex, Toronto, Canada).

Reduction and alkylation

Polypeptides were reduced for 4 h at 37°C under argon in 0.1 M Tris-HCl buffer, pH 8.3, containing 6 M guanidinium chloride and 0.1 M 2-mercaptoethanol and then alkylated at 37°C for 2 h under argon, in the dark, by adding iodoacetamide to a 0.2 M final concentration. After desalting by RP-HPLC on a C8 column (7 µm, 300 Å pore size, 30 × 4.6 mm) using a linear gradient of acetonitrile from 0 to 60% in 0.1% trifluoroacetic acid in 30 min, at a flow rate of 1 ml min^{-1}, the alkylated polypeptides were freeze-dried and dissolved in the appropriate buffer for enzymatic hydrolysis.

Enzymatic hydrolysis

Tryptic hydrolysis was carried out in 0.1 M ammonium bicarbonate pH 8.0 at 37°C for 6 h, using an enzyme-to-substrate ratio of 1:50 (by mass). Chymotryptic digestion was performed in 0.1 M ammonium acetate pH 5.0, at 37°C for 4 h using an enzyme-to-substrate ratio of 1:100 (by mass). Hydrolysis with endoproteinase Glu-C was carried out in 50 mM ammonium acetate pH 4.0, at 25°C for 18 h, using an enzyme-to-substrate ratio of 1:20 (by mass). The enzymatic hydrolysates were fractionated by RP-HPLC on a Nucleosil C18 column (5 µm, 100 Å pore size, 250 × 4.6 mm) using a linear gradient of acetonitrile in 0.1% TFA.

Sequence analysis

Amino acid analyses were performed on a Beckman 6300 amino acid analyzer after hydrolysis in 6 M HCl under vacuum at 110°C for 24 h in the presence of 0.25% phenol. Sequencing of polypeptides, in native or carboxamidomethylated form and of their fragments, was carried out on a gas-phase sequencer (Applied Biosystems 470A) using the 03RPTH program. Phenylthiohydantoin derivatives of amino acids were identified with an on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems 120A).

Digestion of carboxamidomethylated toxins with carboxypeptidase P was performed in 50 mM sodium citrate buffer pH 3.8, at 25°C for 15 min using an enzyme-to-substrate ratio of 1:100 (by mass). The released amino acids were analyzed on the amino acid analyzer.

Neuronal membrane preparations

Rat brain synaptosomes were prepared from adult albino Wistar rats (about 300 g, laboratory bread), according to the procedure of Dodd *et al.* (1981). Insect synaptosomes (P_2L preparation) were prepared from the ventral nervous system of adult cockroach (*Periplaneta americana*) according to established methods (Gordon *et al.*, 1990, 1992; Moskowitz *et al.*, 1994). All buffers contained a cocktail of proteinase inhibitors composed of: phenylmethylsulphonyl fluoride (50 µg ml^{-1}), pepstatin A (1 µM), iodoacetamide (1 mM) and 1 mM of 1,10-phenanthroline. Membrane protein concentration was determined using a Bio-Rad Protein Assay, with BSA as standard.

Radioiodination

Carrier-free $Na^{125}I$ was from Amersham. All other chemicals were of analytical grade. Filters for binding assays were glass fiber GF/C (Whatman, U.K.) preincubated in 0.3% polyethylenimine (Sigma). AaH II was radioiodinated by lactoperoxidase as previously described (Rochat *et al.*, 1977; Cestèle *et al.*, 1995) using 1 nmol of toxin and 1 mCi of carrier free $Na^{125}I$. LqhαIT was iodinated by Iodogen (Pierce Chem. Co. Rockland, U.S.A.) using 5 µg toxin and 0.5 mCi carrier free $Na^{125}I$, as previously described (Gordon and Zlotkin, 1993). The monoiodotoxins were purified according to Lima *et al.* (1989), using a Merck RP C_8 column and a gradient of 5–90% B (A = 0.1% TFA, B = acetonitrile, 0.1% TFA) at a flow rate of 1 ml min^{-1}. The concentrations of the radiolabeled toxins were determined according to the specific activity of the ^{125}I (2424 dpm $fmol^{-1}$ monoiodo-toxin).

Binding assay

Equilibrium competition assays were performed using increasing concentrations of the unlabeled toxin in the presence of a constant low concentration of the radioactive toxin. Standard binding medium composition was: (in mM). Choline Cl 140, $CaCl_2$ 1.8, KCl 5.4, $MgSO_4$ 0.8, HEPES 25 pH 7.4; Glucose 10, BSA 2 mg ml^{-1}. Wash buffer composition was (in mM): Choline Cl 140, $CaCl_2$ 1.8, KCl 5.4, $MgSO_4$ 0.8, HEPES 25 pH 7.4, BSA 5 mg ml^{-1}.

Rat brain synaptosomes (100 μg protein ml^{-1}) or cockroach synaptosomes (P_2L, 3.3 μg ml^{-1}) were suspended in 0.15 ml or 0.3 ml binding buffer, containing ^{125}I-AaH II or ^{125}I-LqhαIT, respectively. After incubation for the designated time periods, the reaction mixture was diluted with 2 ml ice cold wash buffer and filtered through GF/C filters under vacuum. Filters were rapidly washed with an additional 2 × 2 ml buffer. Nonspecific toxin binding was determined in the presence of 0.2 μM unlabeled AaH II or 1 μM LqhαIT, respectively, and consists typically of 15–20% or 1% of total binding for ^{125}I-AaH II or ^{125}I-LqhαIT, respectively. The experiments with the rat brain preparation were carried out for 30 min at 37°C and those with insect membranes, for 60 min at 22°C. Equilibrium competition experiments were analyzed by the iterative computer program LIGAND (Elsevier Biosoft, U.K.). Each experiment was performed at least three times.

In vivo animal bioassays

Fifty percent lethal doses (LD_{50}) were established according to Behrens and Karber (1935). The anti-mammal activity was tested by intra-cerebroventricular (*i.c.v.*) injections into C57 BL/6 mice (20 ± 2 g). Anti-insect activity was evaluated in cockroaches (*Blatella germanica*, 50 ± 2 mg) using an automatic micro-syringe from the Burker Manufacturing Company (Rickmansworth, U.K.).

RESULTS AND DISCUSSION

Purification and sequence analysis of two new scorpion toxins from L. q. hebraeus venom

Each of the ion-exchange fractions 3 and 11 from the venom of the scorpion *Leiurus quinquestriatus hebraeus* (Lqh) yielded a new toxin by fractionation on C18 Nucleosil column, toxins Lqh 3-2 (designated Lqh III, molecular mass: 7048 Da) and Lqh 11-1 (designated Lqh II, molecular mass: 7276 Da), respectively. The toxins were obtained in a high degree of purity as assessed by analytical chromatography (data not show), capillary electrophoresis (Fig. 1, insets), mass spectrometry and direct sequencing of the proteins. The amino acid compositions are presented in Table 1. These toxins contain high amounts of dicarboxylic- and basic residues and of cysteines. Furthermore, Lqh III is characterized by a high content of glycine whereas Lqh II contains a high amount of tyrosine (Table 1).

The data obtained from the automated Edman degradation of the carboxamidomethylated toxins allowed the positive identification of the first 58 and 50 amino acid residues of Lqh II and Lqh III, respectively. The remainder of the sequence of Lqh II was established from the sequence data provided by the carboxy-terminal peptide E-1 generated from the cleavage of the glutamyl bond at position 25 by endoproteinase Glu-C used at pH 4.0 (see Fig. 2). The carboxy-terminal sequence of Lqh III was deduced from data provided by tryptic- and chymotryptic peptides. The presence of a chymotryptic activity in the trypsin resulted in an unexpected cleavage of the Trp(48)–Cys(49) bound, giving rise to peptides T-2a and T-2b. (see Fig. 2). The complete amino acid sequences of the toxins Lqh II and Lqh III are presented in Fig. 2. For both toxins, the sequence data are in good agreement with the data provided by amino acid analysis and mass spectrometry. Moreover, the difference between the calculated molecular mass and the measured molecular mass (Table 1) is indicative of the presence of four disulfide bridges and of the amidation of the C-terminal amino acid residues. The absence of a free α-carboxyl group was confirmed by the failure of carboxypeptidase P to release any amino acid from either Lqh II or Lqh III.

The sequence of the new toxins is compared in Fig. 3 with primary structures of some classical scorpion α- and α-like toxins, as well as with the α-toxins highly active on insects. LqhαIT and Lqq III (Kopeyan *et al.*, 1993; Gordon *et al.*, 1996). The primary structures of Bot III (α-toxin III from the scorpion *Buthus occitanus tunetanus*) and AaH II were previously shown to have three amino acid substitutions (Fig. 3(A)). However, Lqh II reveals only two substitutions as compared to AaH II (96.9% iden-

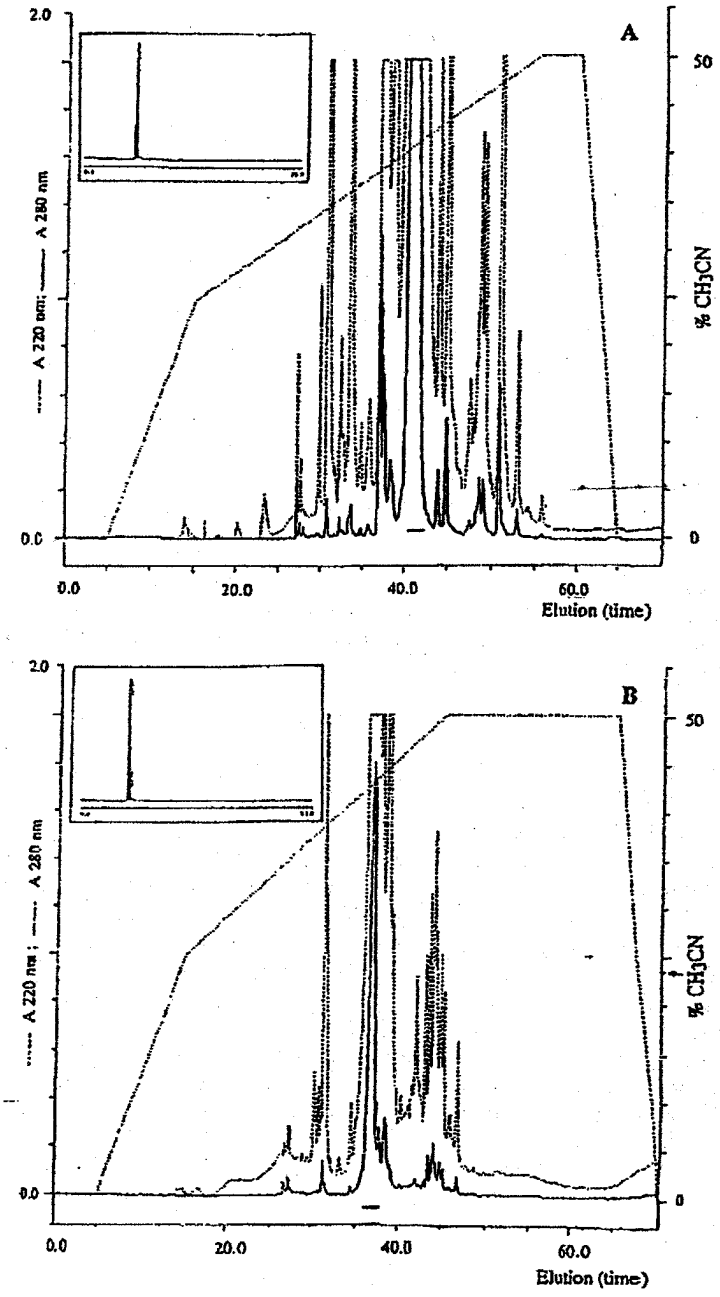


Fig. 1— *Caption on facing page*

Table 1. Amino acid composition of the new *Leiurus* toxins. Molar ratios of amino acids were determined after 24 h acid hydrolysis. Values in parentheses are deduced from the final amino acid sequence. Molecular masses were calculated by taking into account 8 cysteine residues (average mass per residue: 103.14 Da)

Amino acid	Lqh II	Lqh III
Asx	7.8 (8)	6.1 (6)
Thr	2.9 (3)	2.0 (2)
Ser	2.0 (2)	3.9 (4)
Glx	3.8 (4)	4.9 (5)
Pro	3.0 (3)	2.8 (3)
Gly	7.9 (7)	10.6 (11)
Ala	3.4 (3)	3.2 (3)
Cys	8.3 (8)	5.0 (8)
Val	3.1 (3)	4.5 (5)
Met	—	—
Ile	1.6 (2)	1.9 (3)
Leu	2.1 (2)	2.9 (3)
Tyr	5.8 (7)	1.8 (2)
Phe	1.2 (1)	1.9 (2)
His	1.6 (1)	3.7 (4)
Lys	5.1 (5)	3.9 (4)
Arg	3.5 (4)	0.8 (1)
Trp	ND (1)	ND (1)
Total	64	67
Calculated molecular mass (Da)	7285.3	7056.9
Measured molecular mass (Da)	7276.0 ± 0.2	7048.0 ± 0.8

lity), at its first and last amino acid residue, demonstrating the highest identity reported so far for two scorpion toxins derived from two different genus. Lqh III, on the other hand, reveals the highest sequence similarity with the α-like toxin Bom III (Fig. 3(B)), suggesting that it may belong to structural group III. Thus, the structural similarity suggested that Lqh II is a classical scorpion α-toxin and Lqh III belongs to the α-like toxin group (Gordon *et al.*, 1996). We examined their pharmacological activity on both mammals and insects using bioassays and binding studies, to establish their classification and animal selectivity.

Pharmacological activity

Toxicity to mice. The most significant differences among the toxic effects of the various scorpion toxins have been revealed when the toxins were injected directly to mouse

Fig. 1 (*Opposite.*)

Fig. 1. Purification of two new toxins, Lqh 3-2 (Lqh III) and Lqh 11-1 (Lqh II), from the venom of the scorpion *Leiurus quinquestriatus hebraeus* (*Lqh*). (A) Fraction 3, obtained from ion-exchange chromatography of the venom of Lqh, was fractionated by reverse-phase HPLC on a Nucleosil C18 column (for details see Section 2), equilibrated with 0.1% trifluoroacetic acid (TFA). Elution monitored at 220 nm (- - -) and 280 nm (— — —) was performed with a step-wise gradient of acetonitrile in 0.1% TFA (· · ·): 0–25%, 15 min; 25–50%, 40 min, at a flow rate of 2.5 ml h^{-1}. The peak corresponding to toxin Lqh 3-2 (Lqh III) is indicated by a solid bar. Inset: Capillary electrophoresis of toxin Lqh III. Electrophoresis was carried out in 20 mM sodium citrate buffer pH 2.5 at 30 kV and 30°C for 10 min (for details see Section 2). (B) The fraction 11-1 obtained from ion exchange chromatography was fractionated by RP-HPLC as described in (A). For details see Section 2. Elution monitored at 220 nm (- - -) and 280 nm (— — —) was performed with a step-wise gradient of acetonitrile in 0.1% TFA (· · ·): 0–25%, 10 min; 25–50%, 30 min, at a flow rate of 2.5 ml h^{-1}. The peak corresponding to toxin Lqh 11-1 (Lqh III) is indicated by a solid bar. Inset: Capillary electrophoresis of toxin Lqh II. Electrophoresis was carried out as described in (A).

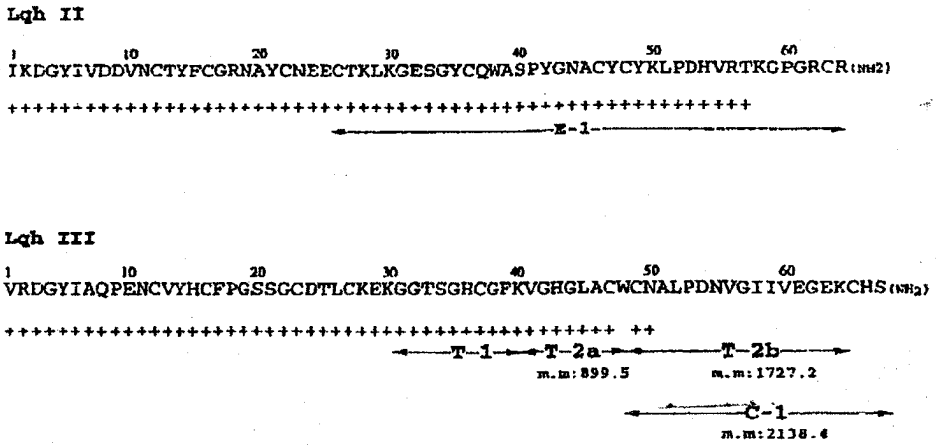


Fig. 2. Amino acid sequences of toxins Lqh II (Lqh II-1) (A) and Lqh III (Lqh 3-2) (B) from the venom of the scorpion *Leiurus quinquestriatus hebraeus*. Methods used for the determination of the sequence (see Section 2) are indicated as follows: + + + automated Edman degradation of the toxin. T-, C- and E- indicate tryptic peptides, chymotryptic peptides and peptide generated from cleavage of the toxin with endoproteinase Glu-C, respectively.

brain, by the intracerebroventricular route (see Martin-Eauclaire *et al.*, 1992 for references; Gordon *et al.*, 1996). The toxin Lqh II is highly toxic to mice, having LD_{50} of 1.9 ng/20 g mouse (Table 2). This value is similar to the previously published LD_{50} of the most active scorpion α-toxin on mammals, AaH II (0.5-1 ng/20 g mouse; see Martin-Eauclaire *et al.*, 1992). AaH II is 2200-fold more toxic to mice than Lqq III, the scorpion α-toxin highly active on insects, shown to share receptor site 3 with LqhαIT on insect sodium channels (Gordon *et al.*, 1996; Cestele *et al.*, 1997). Thus, the new toxin from Lqh venom, Lqh II, reveals toxicity to mice comparable to that of AaH II, suggesting that the two substitutions in its amino acid sequence are not impairing its anti-mammalian activity (Table 2).

The second new toxin, Lqh III, shown to have the highest sequence identity with Bom III (80.6% of identity, Table 2), reveals about 2-fold lower anti-mammal toxicity than Bom III and Bom IV (see Table 2; Martin-Eauclaire *et al.*, 1992; Gordon *et al.*, 1996). The most active scorpion α-toxins on insects (LqhαIT and Lqq III) reveal relatively very low toxicity to mice by the intracerebroventricular route (Table 2; but relatively high toxicity by subcutaneous route: Gordon *et al.*, 1996; Cestele *et al.*, 1997).

Toxicity to cockroach. To examine the selectivity of these new toxins to mammals vs insects, they have been injected to adult cockroaches (*Blatella germanica*), previously shown to be highly sensitive to various scorpion and sea anemone toxins (Gordon *et al.*, 1996). Lqh II revealed 3.2-fold higher toxicity to insects, as compared to AaH II (Table 2), while Lqh III was 32.3-fold more active to cockroach than AaH II, revealing comparable anti-insect toxicity to Bom IV and 1.9-fold more toxic than Bom III (Table 2).

A.

```
II  Lqh II    -IKDGYIVDDVNCTYFCGR-NAYCNEECTKLKG-ESGYCQWASPYGNACYCYK-LP-DHVRTKGPGR-CR-
    AaH II    -V.........C...C..-...C...C.....-....C.........C.C..-..-..........-CH-
    Bot III   -V........R.C...C..-...C...C.....-....C.........C.C..-V.-..........-CN
    Lqq V     -L........K.C.F.C..-...C.D.CK.K..-....C.........CWC..-..-.R.SI.EK..-CN-

III Lqh III   -VRDGYIAQPENCVYHCFPGSSGCDTLCKEK-GGTSGHCGFKVGHGLACWCNA-LP-DNVGIIVEGEKCHS
    Bom III   -G.........C...C......C...C....-.A....C..LP.S.V.C.C-DN..-NK.P.V.G...C.-

    Lqq III   -...A...KNY.C..EC.R-D.YCND.CTKN-.AS..YCQWAGKY.N.C.CY.-..-...P.R.P.-.C.-
    LqhαIT    -...A...KNY.C..EC.R-DAYCNE.CTKN-.AS. YCQWAGKY.N.C.CY.-..-...P.R.P.-.CR-

IV  Lqq IV    G...A...DDK.C..TCGS-N.YCN.ECTKN-.AE..YCQWLGKY.N.C.CIK-..-.K.P.RIP.-.CR-
```

B.

TOXIN	Lqh II	AaH II	Lqq V	Lqh III	Bom III	LqhαIT	Lqq III	Lqq IV
Lqh II	100							
AaH II	96.9	100						
Lqq V	81.2	81.2	100					
Lqh III	40.6	46.9	44.8	100				
Bom III	33.3	34.8	42.4	80.6	100			
LqhαIT	60.9	62.5	59.4	53.7	50.0	100		
Lqq III	54.7	57.8	57.8	59.7	50.8	95.3	100	
Lqq IV	62.0	61.5	60.0	50.7	52.3	73.8	73.2	100

Fig. 3. Comparison of scorpion toxin amino acid sequences classified according to their structural homology. (A) Comparison of the amino acid sequence of Lqh II and Lqh III, taken as references, with some scorpion toxins that affect sodium current inactivation. Only non identical amino acids and the conserved cysteine residues are shown for the clarity of presentation. Single letter code for amino acids is used. (.) indicates identical residues and (–) are deletions introduced for alignment of sequences. The structural group is marked on the left (II–IV). For references for the sequences see Martin-Eauclaire and Couraud (1995). (B) Percentage of identical residues calculated for maximum homology between each pair of protein sequence.

Binding studies. We have previously suggested several criteria for a scorpion toxin to belong to the so-called α-like group; sequence resemblance; to inhibit sodium current inactivation; being active on mammals or both mammals and insects but *not* to compete with the classical scorpion α-toxin AaH II for binding to receptor site 3 on rat brain sodium channels (Gordon *et al.*, 1996; Gordon *et al.*, in press). Using these criteria, Bom III and Bom IV have been considered to represent the new α-like group (Gordon *et al.*, 1996). Moreover, α-like scorpion toxins were shown to compete for binding with the most active scorpion α-toxins on insects, LqhαIT and Lqq III (Gordon *et al.*, 1996; Cestele *et al.*, 1997). On this background we examined the binding of the new toxins in competition binding assays for the binding of ^{125}I-AaH II and ^{125}I-LqhαIT in rat brain and cockroach synaptosomes, respectively.

Figure 4 presents competition studies of Lqh II and Lqh III for scorpion α-toxin binding in rat brain (A) and cockroach (B) neuronal membrane. Lqh II reveals IC_{50} values (Table 2) comparable to that of AaH II, confirming its high anti-mammal tox-

Table 2. Activity of some scorpion α- and α-like toxins in mice and insect

Toxin	LD_{50} mice (i.c.v)* (ng/20 g^{-1})	LD_{50} mice (s.c.)† (pmol g^{-1})	LD_{50} cockroach‡ (pmol g^{-1})	IC_{50} (nM) Rat brain (^{125}I-AaH II)§	IC_{50} (nM) Cockroach (^{125}I-LqhαIT)¶
AaH II	0.5‖	1.7‖	897**	0.2‖	58.8 ± 12.6**
Lqh II	1.9	n.d.	280	0.4 ± 0.1	5.72 ± 0.63
Lqh III	50	23	28	> 1000	0.43 ± 0.20
Bom III	23‖	19.7	52.6**	≫1000‖ ††	29.3 ± 8.1**
Bom IV•	23‖	5.5‖	19.7**	≫1000‖ ††	4.6 ± 1.6**
LqhαIT	n.d.‡‡	8.3**	2.5**	> 1000** ††	0.02 ± 0.01**
Lqq III	1100‖	6.9	8.3**	700‖	0.03 ± 0.01**

* Intracerebroventricular injection.
† Subcutaneous injection.
‡ Injection to abdominal segments of *Blatella germanica*, 50 ± 2 mg body weight.
§ Competition for ^{125}I-AaH II binding in rat brain synaptosomes.
¶ Competition for ^{125}I-LqhαIT binding in cockroach synaptosomes.
‖ Martin-Eauclaire *et al.*, 1992.
** Gordon *et al.*, 1996.
†† No significant inhibition was detected at 1 μM toxin.
‡‡ Not determined.

icity and similarity in action to AaH II. In contrast, Lqh III is a very poor competitor for AaH II binding, revealing a partial displacement at very high concentration. The lack of interaction between the binding of Lqh III and that of AaH II (known to bind to receptor site 3) is similar to the previously described situation with Bom III and Bom IV (Table 2; Martin-Eauclaire *et al.*, 1992; Gordon *et al.*, 1996). Thus, the Lqh III receptor site is distinct from receptor site 3 on rat brain sodium channels.

Conversely, both toxins, Lqh II and Lqh III, are able to displace ^{125}I-LqhαIT from its binding site on cockroach sodium channels (Fig. 4(B)). The IC_{50} for Lqh III and Lqh II is about 22- and 286-fold higher than that of LqhαIT, respectively, and 68- and 5.1-fold lower than the IC_{50} of Bom III (Table 2). The ability of both toxins to bind with apparent high affinity (at the nanomolar range) to cockroach sodium channels is in accordance with their increased anti-insect activity (Table 2).

The anti-mammal as well as anti-insect activity of these toxins indicate that relatively minor changes in the sequence of scorpion toxins may change their relative selectivity. This point is specially emphasized by the minor substitutions revealed between AaH II and Lqh II primary structures (Figs 2 and 3). The significant increase in anti-insect activity in Lqh II as compared to AaH II (3.2-times in toxicity and 10.3-times in apparent affinity, Table 2), may be attributed mainly to the C-terminal substitution (from His to Arg, see Fig. 3), since the N-terminal residue (the second substitution) has been suggested to be burried in the toxin three dimentional structure (Tugarinov *et al.*, 1997; Gordon *et al.*, in press). Interestingly, similar substitution of the C-terminal residue is also observed between Lqq III and LqhαIT (Fig. 3). Thus, we suggest that arginine residue (or additional positive charge) at the C-terminal end of scorpion α-toxins may participate in anti-insect activity, since Lqq III has been shown to be more active on mammals and less active on insects, as compared to LqhαIT (Table 2; Gordon *et al.*, 1996; Cestele *et al.*, 1997). In contrast, positive charge at the C-terminus is not required for anti-mammal activity, since none of the highly active scorpion α-toxins on vertebrates have positively charge residue at this position (see Fig. 3 and Gordon *et al.*, in press).

Thus, Lqh II may serve as a specific probe for receptor site 3 on both mammalian and insect sodium channels. The unusual identity in its primary structure to the most

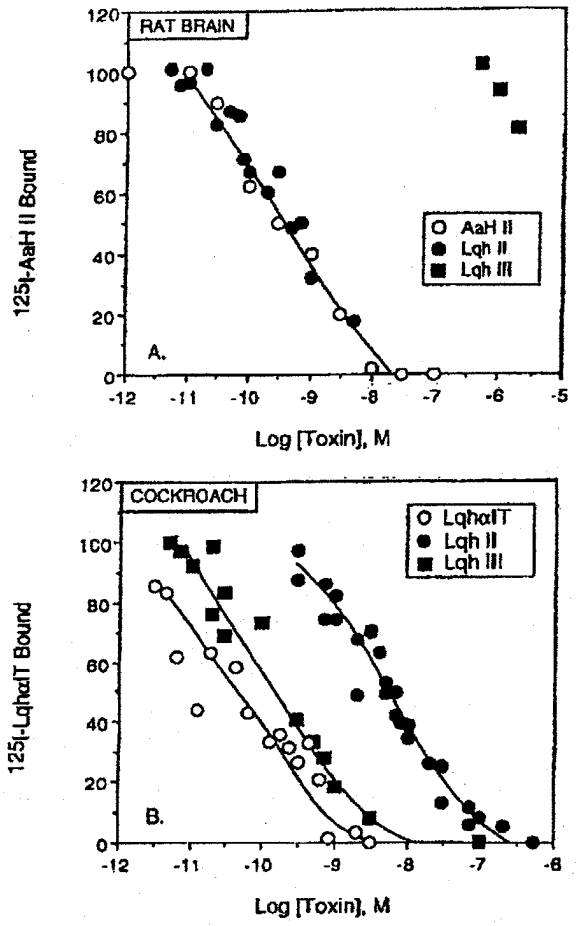


Fig. 4. Competition for ^{125}I-scorpion α-toxin binding by Lqh II and Lqh III toxins in rat brain and cockroach synaptosomes. The amount of ^{125}I-α-toxin bound is expressed as the percentage of the maximal specific binding in the system without additional toxins. All curves were analyzed by the LIGAND program and IC_{50} values were calculated using DRUG analysis and are shown in Table 2. The lines are drawn by hand. (A) Competition for ^{125}I-AaH II in rat brain synaptosomes. Rat brain synaptosomes were incubated for 30 min at 37°C in the presence of 0.2 nM ^{125}I-AaH II and increasing concentrations of cold AaH II, Lqh II and Lqh III, as described in Section 2. Non specific binding, determined in the presence of 200 nM AaH II, was subtracted. (B) Competition for ^{125}I-LqhαIT binding. Cockroach neuronal membranes (1–2 μg protein) were incubated for 60 min at 22°C in the presence of 50–60 pM of ^{125}I-LqhαIT and increasing concentrations of LqhαIT, Lqh II and Lqh III. Nonspecific binding, determined in the presence of 1 μM LqhαIT, was subtracted from all data points.

active scorpion α-toxin on mammals makes it a valuable tool to study the structure of the receptor site for scorpion α-toxins in excitable tissues. The second new toxin, Lqh III, belongs to the α-like scorpion toxins by structure comparison; its high activity on both mammals and insects and by its binding profile. Lqh III is a very poor competitor

for AaIT II binding in rat brain, but reveals apparent high affinity to insect neuronal membranes, as previously shown for Bom III and Bom IV (Gordon *et al.*, 1996). This is the first toxin belonging to α-like group described from Lqh venom. The high toxicity of Lqh III to both mammals and insects may be useful in understanding the structural basis for animal group selectivity of scorpion toxins.

Acknowledgements We are grateful to Prof Herve Rochat, Laboratory of Biochemistry, CNRS URA 1455, Faculty of Medicine Nord, Marseille, France, for his support, his useful remarks on the manuscript and for kindly providing the AaH II toxin; to Prof. Michael Gurevitz and Noam Zilberberg, Department of Botany, Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel, for the generous gift of the recombinant LqhaIT, and to Prof. Andre Tartar, URA 1309 CNRS Service de Chimie des Biomolecules, Institut Pasteur, Lille, France, for his support and encouragement.

REFERENCES

Behrens, B. and Karber, C. (1935) Wie sind reicheuversuche fur biologische auswertungen am zweckmassigsten anzuordnen? *Arch. Exp. Pathol. Pharmak.* **177**, 379–388.

Catterall, W. A. (1980) Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu. Rev. Pharmacol. Toxicol* **20**, 15–43.

Catterall, W. A. (1986) Molecular properties of voltage-sensitive sodium channels. *Annu. Rev. Biochem.* **55**, 953–985.

Catterall, W. A. (1992) Cellular and molecular biology of voltage-gated sodium channels. *Pharm. Rev.* **72** (Suppl.)(4), 15–48.

Catterall, W. A. and Beress, L. (1978) Sea anemone toxin and scorpion toxin share a common receptor site associated with the action potential sodium ionophore. *J. Biol. Chem.* **253**, 7393–7396.

Cestele, S., Ben Khalifa, R., Pelhate, M., Rochat, H. and Gordon, D. (1995) α-Scorpion toxins binding on rat brain and insect sodium channels reveal divergent allosteric modulations by brevetoxin and veratridine. *J. Biol. Chem.* **270**, 15153–15161.

Cestele, S., Gordon, D., Kopeyan, C. and Rochat, H. (1997) Toxin III from *Leiurus quinquestriatus quinquestriatus*: a specific probe for receptor site 3 on insect sodium channels. *Insect Biochem. Molec. Biol.* **27**(6), 523–528.

Couraud, F., Rochat, H. and Lissitzky, S. (1978) Binding of scorpion and sea anemone neurotoxins to a common site related to the action potential Na^+ ionophore in neuroblastoma cells. *Biochem. Biophys. Res. Commun.* **83**, 1525–1530.

Delori, P., Van Rietschoten, J. and Rochat, H. (1981) Scorpion venoms and neurotoxins: an immunological study. *Toxicon* **19**, 393–407.

Dodd, P. R., Hardy, J. A., Oakley, A. E., Edwardson, J. A., Perry, E. K. and Delaunoy, J. P. (1981) A rapid method for preparing synaptosomes: comparison, with alternative procedures. *Brain Research* **226**, 107–118.

Dufton, M. J. and Rochat, H. (1984) Classification of scorpions toxins according to amino acid composition and sequence. *J. Mol. Evol.* **20**, 120–127.

Eitan, M., Fowler, E., Herrmann, R., Duval, A., Pelhate, M. and Zlotkin, E. (1990) A scorpion venom neurotoxin paralytic to insects that affects sodium current inactivation: purification, primary structure, and mode of action. *Biochemistry* **29**, 5941–5947.

Gordon, D. (1997a) Sodium channels as targets for neurotoxins: mode of action and interaction of neurotoxins with receptor sites on sodium channels. In *Toxins and Signal Transduction*, Cellular and Molecular Mechanisms of Toxin Action Series, ed. Y. Gutman and P. Lazarowici. Harwood Academic Press, pp. 119–149.

Gordon, D. (1997b) Molecular biology of voltage-gated ionic channels: Structure-function relationships. In *Membranes and Cell Signaling*, Principles of Medical Biology, Vol. 7A, ed. E. E. Bitter and N. Bitter, pp. 245–305. JAI Press, Greenwich, CT, U.K.

Gordon, D. and Zlotkin, E. (1993) Binding of an alpha scorpion toxin to insect sodium channels is not dependent on membrane potential. *FEBS Lett.* **315**, 125–129.

Gordon, D., Jover, E., Couraud, F. and Zlotkin, E. (1984) The binding of an insect selective neurotoxin (AaIT) from scorpion venom to locust synaptosomal membranes. *Biochim. Biophys. Acta* **778**, 349–358.

Gordon, D., Merrick, D., Wollner, D. A. and Catterall, W. A. (1988) Biochemical properties of sodium channels in a wide range of excitable tissues studied with site-directed antibodies. *Biochemistry* **27**, 7032–7038.

Gordon, D., Moskowitz, H. and Zlotkin, E. (1990) Sodium channel polypeptides in central nervous systems of various insects identified with site-directed antibodies. *Biochim. Biophys. Acta* **1026**, 80–86.

Gordon, D., Moskowitz, H., Eitan, M., Warner, C., Catterall, W. A. and Zlotkin, E. (1992) Localization of receptor sites for insect-selective toxins on sodium channels by site-directed antibodies. *Biochemistry* **31**, 7622–7628.

Gordon, D., Moskowitz, H. and Zlotkin, E. (1993) Biochemical characterization of insect sodium channels. *Arch. Insect Biochem. Physiol.* **22**, 41–53.

Gordon, D., Martin-Eauclaire, M. F., Cestele, S., Kopeyan, C., Carlier, E., Ben Khalifa, R., Pelhate, M. and Rochat, H. (1996) Scorpion toxins affecting sodium current inactivation bind to distinct homologous receptor sites on rat brain and insect sodium channels. *J. Biol. Chem.* **271**, 8034–8045.

Gordon, D., Savarin, P., Gurevitz, M. and Zinn-Justin, S. (1998) Functional anatomy of scorpion toxins affecting sodium channels. *J. Toxicol. Toxin Rev.* (in press).

Jover, E., Martin-Moutot, N., Couraud, F. and Rochat, H. (1978) Scorpion toxin specific binding to rat synaptosomes. *Biochem. Biophys. Res. Commun.* **85**, 377–382.

Kharrat, R., Darbon, H., Rochat, H. and Granier, C. (1989) Structure-activity relationships of scorpion α-toxins. Multiple residues contribute to the interaction with receptors. *Eur. J. Biochem.* **181**, 381–390.

Kopeyan, C., Mansuelle, P., Martin-Eauclaire, M. F., Rochat, H. and Miranda, F. (1993) Characterization of toxin III of the scorpion *Leiurus quinquestriatus quinquestriatus*: a new type of alpha-toxin highly toxic both to mammals and insects. *Natural Toxins* **1**, 308–312.

Lima, M. E., Martin-Eauclaire, M. F., Hue, B., Loret, E., Diniz, C. R. and Rochat, H. (1989) On the binding of two scorpion toxins to the central nervous system of the cockroach *Periplaneta americana*. *Insect Biochem.* **19**, 413–422.

Loughney, K., Kreber, R. and Ganetzky, B. (1989) Molecular analysis of the para locus, a sodium channel gene in *Drosophila*. *Cell* **58**, 1143–1154.

Marshall, D. L., Vatanpour, H., Harvey, A. L., Boyot, P., Pinkasfeld, S., Doljansky, Y., Bouet, F. and Menez, A. (1994) Neuromuscular effects of some potassium channel blocking toxins from the venom of the scorpion *Leiurus quinquestriatus hebraeus*. *Toxicon* **32**, 1433–1443.

Martin-Eauclaire, M. F. and Couraud, F. (1995) Scorpion neurotoxins: effects and mechanisms. In *Handk. Neurotoxicology*, ed. L. W. Chang and R. S. Dyer, pp. 683–716. Marcel Dekker, New York.

Martin-Eauclaire, M. F., Delabre, M. L., Ceard, B., Ribeiro, A. M., Sogaard, M., Svensson, B., Diniz, C. R., Smith, L. A., Rochat, H. and Bougis, P. E. (1992) Genetics of scorpion toxins. In *Recent Advances in Toxinology Research*, Vol. 1, ed. P. Gopalakrishnakone and C. K. Tan, pp. 196–209. Venom and Toxin Research Group, National University of Singapore, Singapore.

Miranda, F., Kopeyan, C., Rochat, H., Rochat, C. and Lissitzky, S. (1970) Purification of animal neurotoxins. Isolation and characterization of eleven neurotoxins from the venoms of the scorpion *Androctonus australis* Hector, *Buthus occitanus tunetanus*, and *Leiurus quinquestriatus quinquestriatus*. *Eur. J. Biochem.* **16**, 514–523.

Moskowitz, H., Herrmann, R., Zlotkin, E. and Gordon, D. (1994) Variability among insect sodium channels revealed by binding of selective neurotoxins. *Insect Biochem. Molec. Biol.* **24**, 13–19.

Moskowitz, H., Zlotkin, E. and Gordon, D. (1991) Solubilization and characterization of the insect neuronal sodium channel. *Neurosci. Lett.* **124**, 148–152.

Pelhate, M. and Sattelle, D. B. (1982) Pharmacological properties of insect axons: a review. *J. Insect. Physiol.* **28**, 889–903.

Pelhate, M. and Zlotkin, E. (1982) Actions of insect toxin and other toxins derived from the venom of the scorpion *Androctonus australis* on the isolated giant axons of the cockroach (*Periplaneta americana*). *J. Exp. Biol.* **97**, 67–71.

Possani, L. D. (1984) Structure of scorpion toxins. In *Insect, Poisons, Allergens and Other Invertebrate Venoms*, ed. T. Tu, pp. 513–550. Handk. Nat. Toxins 2.

Rochat, C., Tessier, M., Miranda, F. and Lissitzky, S. (1977) Radioiodination of scorpion and snake neurotoxins. *Anal. Biochem.* **82**, 532–548.

Rochat, H., Bernad, P. and Couraud, F. (1979) Scorpion toxins: chemistry and mode of action. In *Advances in Cytopharmacology*, Vol. 3, ed. B. Ceccarelli and F. Clementi, pp. 325–334. Raven Press, New York.

Rogers, J. C., Qu, Y., Tanada, T. N., Scheuer, T. and Catterall, W. A. (1996) Molecular determinants of high affinity binding of α-scorpion toxin and sea anemone toxin in the S3–S4 extracellular loop in domain IV of the sodium channel α subunit. *J. Biol. Chem.* **271**, 15950–15962.

Strichartz, G., Rando, T. and Wang, G. K. (1987) An integrated view of the molecular toxicology of sodium channel gating in excitable cells. *Annu. Rev. Neurosci.* **10**, 237–267.

Trainer, V. L., McPhee, J. C., Boutelet-Bochan, H., Baker, C., Scheuer, T., Babin, D., Demoute, J. P., Guedin, D. and Catterall, W. A. (1997) High affinity binding of pyrethroids to the α subunit of brain sodium channels. *Mol. Pharmacol.* **51**, 651–657.

Tugarinov, V., Kustanovich, I., Zilberberg, N., Gurevitz, M. and Anglister, J. (1997) Solution structure of a highly insecticidal recombinant scorpion α-toxin and a mutant with increased activity. *Biochemistry* **36**, 2414–2424.

Watt, D. D. and Simard, J. M. (1984) Neurotoxic proteins in scorpion venom. *J. Toxicol.* **3**, 181–221.

Zilberberg, N., Gordon, D., Pelhate, M., Adams, M. E., Norris, F., Zlotkin, E. and Gurevitz, M. (1996) Functional expression and genetic modification of an alpha scorpion neurotoxin. *Biochemistry* **35**, 10215–10222.

Zlotkin, E. (1986) The interaction of insect selective neurotoxins from scorpion venoms with insect neuronal membranes. In *Neuropharmacology and Pesticides Action*, ed. M. G. G. Lunt, R. C. Raey and P. N. R. Usherwood, pp. 352–383. Ellis Horwood, Weinheim.

Zlotkin, E., Eitan, M., Bindokas, V., Adams, M. E., Moyer, M., Brukhart, W. and Fowler, E. (1991) Functional duality and structural uniqueness of depressant insect-selective neurotoxins. *Biochemistry* **30**, 4814–4820.

Zlotkin, E., Kadouri, D., Gordon, D., Pelhate, M., Martin, M. F. and Rochat, H. (1985) An excitatory and a depressant insect selective toxin from scorpion venom both affect sodium conductance and possess a common binding site. *Arch. Biochem. Biophys.* **240**, 877–887.

Zlotkin, E., Miranda, F. and Rochat, H. (1978). Chemistry and pharmacology of Buthinae scorpion venoms. In *Arthropods Venoms*, ed. S. Bettini, pp. 317–369. Springer-Verlag, Berlin.

Eur. J. Biochem. 254, 44–49 (1998)

A depressant insect-selective toxin analog from the venom of the scorpion *Leiurus quinquestriatus hebraeus*

Purification and structure/function characterization

Haim MOSKOWITZ[1], Rafael HERRMANN[1], A. Daniel JONES[2] and Bruce D. HAMMOCK[1]

[1] Departments of Entomology and Environmental Toxicology, University of California, Davis, USA
[2] Facility for Advanced Instrumentation, University of California, USA

(Received 12 September 1997/2 February 1998) – EJB 97 1307/3

The scorpion venom-derived excitatory and depressant insect-selective polypeptide neurotoxins modify sodium conductance in insect neuronal membranes and differ greatly in their primary structures and symptoms induced in blow fly larvae. We report here the purification and characterization of a new insect selective toxin, $LqhIT_5$. $LqhIT_5$ is more similar to the excitatory toxins in its mode of action and the depressant toxins in its primary structure. This toxin is a single polypeptide composed of 61 amino acids that are cross linked by four disulfide bonds. When $LqhIT_5$ is injected into blow fly larvae, a fast contraction paralysis occurs without depressant activity. No mammalian toxicity was detected by subcutaneous or intracranial injections of this toxin into mice. Sequence comparison of $LqhIT_5$ and known depressant toxins shows a high degree of similarity among the amino acids located on the C-terminus of the toxins. However, there are some clear differences in the amino acids located close to the N-terminus of the toxins. By the aid of homology modeling, we demonstrated that these amino acids have the same orientation in the tertiary structure of the molecule and are exposed to the environment. The change in the mode of action of $LqhIT_5$ (no depressant activity) by substitutions of a few amino acids located on a specific exposed area of the toxin shed a new light on the structure/function relationship of scorpion toxins. These results caution that similarity in the mechanism of action of scorpion toxins does not always follow from an overall similarity in sequence.

Keywords: scorpion toxins; depressant insect toxin; mode of action.

Scorpion venoms contain a number of polypeptide toxins that specifically block or alter gating properties of ion channels. Among those toxins are the small polypeptides (30–40 amino acids) with three or four disulfide bridges which mainly affect K^+ channels [1, 2] and the large polypeptides (60–70 amino acids) with four disulfide bridges that mainly affect sodium channels [3–5]. The latter group of toxins can be subdivided into mammalian toxins [4, 6] which are responsible for human envenomation, and the insect-selective toxins which are shown to modify the sodium conductance exclusively in insect neuronal membranes [7, 8]. The insect-selective toxins are subdivided into two categories which can easily be distinguished according to their effect on blow fly larvae and their amino acid sequences (Table 1). One category of the insect-selective toxins include the depressant toxins which, so far, are composed of 61 amino acids and induce a slow progressive onset of flaccid paralysis preceded by a short transient phase of contractility [5]. The other category of the insect-selective toxins is the excitatory toxins which, so far, are composed of 70 amino acids and induce an immediate reversible fast contraction paralysis upon injection [3]. There is a high sequence similarity among the toxins in each category, however, the similarity between categories is much lower [5]. Recently, new depressant toxin analogs (analogous in sequence but not in activity) named $BotIT_2$ and $AaIT_5$ were purified from the venoms of the scorpions *Buthus occitanus tunetanus* and *Androctonus australis* Hector, respectively. $BotIT_2$ was shown to have high sequence similarity to the depressant insect toxins. However, an excitatory activity in insects and some mammalian toxicity were also reported [9]. $AaIT_5$ also showed high sequence similarity to the depressant toxins but did not show mammalian toxicity upon injection into mice. No depressant or excitatory activity was reported for $AaIT_5$ when injected

Correspondence to B. D. Hammock, Departments of Entomology and Environmental Toxicology, University of California, Davis, CA 95616, USA

Abbreviations. AaIT, excitatory insect toxin derived from the venom of the scorpion *Androctonus australis*; $AaIT_5$, insect toxin derived from the venom of the scorpion *Androctonus australis*; $BaIT_2$, a depressant insect-selective toxin derived from the venom of the scorpion *Buthacus arenicola*; $BotIT_2$, toxin derived from the venom of the scorpion *Buthus occitanus tunetanus*; CPU, dose that causes a contractile paralysis to 50% of the animals; FPU, dose that causes a flaccid paralysis to 50% of the animals; LqhαIT, α insect toxin derived from the venom of the scorpion *Leiurus quinquestriatus hebraeus*; $LqhIT_2$, depressant insect-selective toxin derived from the venom of the scorpion *Leiurus quinquestriatus hebraeus*; $LqhIT_5$, excitatory insect-selective toxin derived from the venom of the scorpion *Leiurus quinquestriatus hebraeus*; LqhIV, toxin derived from the venom of the scorpion *Leiurus quinquestriatus hebraeus*; $LqqIT_1$, excitatory insect-selective toxin derived from the venom of the scorpion *Leiurus quinquestriatus quinquestriatus*; $LqqIT_2$, depressant insect-selective toxin derived from the venom of the scorpion *Leiurus quinquestriatus quinquestriatus*; LqqIII, LqqIV, α mammalian toxins derived from the venom of the scorpion *Leiurus quinquestriatus quinquestriatus*.

Note. The novel amino acid sequence mentioned in this paper has been deposited with the Protein Identification Resource and European Bioinformatics Institute (EBI) data bases and are available under accession numbers A59006 and P81240, respectively.

into larvae of the blow fly. Moreover, this toxin showed high toxicity to larvae of *Lepidoptera* but very low toxicity to blow fly larvae [10].

We report here the purification and characterization of a new excitatory insect-selective toxin from the venom of the scorpion *Leiurus quinquestriatus hebraeus* ($LqhIT_5$). This toxin is similar to the excitatory toxins in its action and similar to the depressant toxins in its primary structure. $LqhIT_5$ is 61 amino acids and has high sequence similarity to the known depressant insect-selective toxins but does not show the depressant activity of flaccid paralysis. In the present study, the importance of the change in the mode of action without changing the selectivity of the toxin towards insects is discussed.

EXPERIMENTAL PROCEDURES

Venom. Venom of the scorpion *Leiurus quinquestriatus hebraeus* was obtained from Sigma Chemical Co.

Test animals. Larvae of the blow fly *Sarcophaga falculata* were bred in our laboratory as previously described [11]. Swiss Webster mice (three weeks of age) were obtained from Charles River and were housed according to approved animal care protocols.

Bioassays. For the data reported in this study, the toxicity of $LqhIT_5$ was determined on the material following microbore HPLC purification and its purity evaluated by other analytical methods including capillary electrophoresis, isoelctric focusing, mass spectrometry and a single amino acid in the first cycle of amino acid sequencing. Three replicates of 25–40 blow fly (*S. falculata*) larvae each were injected with each of the toxins. The dose causing contractile paralysis in 50% of the animals (CPU) was determined (using the method developed by Reed and Muench [12]) as that needed for a fast contraction paralysis 1-min post-injection of the toxin into an abdominal intersegmental membrane, and the dose causing a flaccid paralysis in 50% of the animals (FPU) was determined as that needed for a progressive flaccid paralysis 5-min post-injection. The symptoms induced by depressant insect-selective toxin derived from the venom of *L. quinquestriatus quinquestriatus* ($LqhIT_2$) and excitatory insect toxin derived from the venom of *Androctonus australis* (AaIT) were used for comparison with a standard depressant and standard excitatory toxin, respectively. The toxicity towards mice was evaluated 24 hours after subcutaneous or intracranial injection of the toxin. Mice were monitored for symptoms throughout the 24-hour period.

Column chromatography. Lyophilized venom (50 mg) of *L. quinquestriatus hebraeus* was homogenized in 2 ml 10 mM ammonium acetate, pH 6.4, and the insoluble material removed by centrifugation at 27000 *g* for 20 min. The supernatant was collected and the pellet was resuspended in 2 ml 10 mM ammonium acetate, pH 6.4, homogenized and centrifuged again. This extraction was repeated four times to maximize the yield of protein extracted from the venom. The supernatant from all the extractions was loaded on a low-pressure cation-exchange column (10 ml), using CM-52 cellulose (Whatman) equilibrated in 10 mM ammonium acetate, pH 6.4, at a flow rate of 10 ml/hour. The fraction that did not bind to the cation-exchange column was further fractionated by HPLC (Perkin Elmer Series 410 pump) reverse-phase chromatography using a Vydac C_4 column (4.6 mm×250 mm). The column was equilibrated in 5% acetonitrile containing 0.1% trifluoroacetic acid (buffer A), and the fraction was loaded onto the column and eluted with a linear gradient of 0–60% buffer B for 70 min at a flow rate of 0.6 ml/min. Buffer B was 95% acetonitrile containing 0.1% trifluoroacetic acid. The fractions were monitored at 214 nm on a Perkin Elmer diode array detector which was controlled by Perkin Elmer Omega-4 software. The final step of purification was carried out using a Reliasil C_{18} reverse-phase column on a microbore HPLC (Michrom Bioresources Inc.). The column was equilibrated in buffer A. The toxic fraction (as determined by injection into blow fly larvae) that was obtained by the reverse-phase C_4 chromatography was loaded onto the C_{18} column and eluted in a linear gradient of 0–60% buffer B over 60 min at a flow rate of 50 µl/min. Absorbency was monitored at 214 nm and peaks were collected accordingly.

Purity determination. Following two HPLC steps, the homogeneity and purity of the toxin was evaluated by several techniques including free solution capillary electrophoresis (Applied Biosystems model 270A). The 75-cm uncoated fused-silica capillary was equilibrated for 4 min with 20 mM sodium citrate, pH 2.9, and the toxin (0.2 mg/ml) was loaded using vacuum for 2 s. The running buffer was 20 mM sodium citrate, pH 2.9, the electric potential was 20 kV and the absorbance was monitored at 214 nm. Peak areas were integrated. The purity of the toxin was also evaluated by analytical isoelectric focusing (IEF) utilizing cast polyacrylamide gels (Ampholine PAGplate pH 3.5–9.5; Pharmacia Biotech) and pI markers in the range 3.5–9.3 (Pharmacia Biotech). Proteins were visualized by staining with Coomassie brilliant blue G-250 [13].

Sequence determination. $LqhIT_5$ was reduced and alkylated by incubation in 6 M guanidine hydrochloride, 0.1 M Tris/HCl, pH 8.3, containing 1 mM EDTA and 20 mM dithiothreitol for 1 hour at 37°C. Iodoacetic acid was added to a final concentration of 50 mM and incubated for an additional 1 hour at 37°C in the dark. The N-terminal sequence of the reduced and alkylated toxin was determined using a HP GS1000 sequence analyzer by automated Edman degradation (in the Protein Structure Lab at U. C. Davis, USA).

Mass spectrometry. The molecular mass of $LqhIT_5$ was determined using electrospray mass spectrometry on a VG/Fisons Quattro-BQ mass spectrometer (VG Biotech). An Isco µLC-500 syringe pump delivered the mobile phase (acetonitrile/water, 50:50, by vol.) at 5 µl/min. Solutions of purified peptides were analyzed by direct flow injection using an injection volume of 10 µl. Spectra were obtained in positive-ion mode using a capillary voltage of + 3.5 kV, a cone voltage of 50 V and a source temperature of 65°C. Spectra were scanned over the range 500–1500 *m/z* at a rate of 20 s/scan; 20 scans were combined using the VG MCA acquisition mode. Molecular masses were determined using the maximum entropy deconvolution algorithm (MaxEnt) to transform the *m/z* range 650–1500 to give a true mass scale spectrum. Mass calibration was performed using horse heart myoglobin (Sigma Chemical Co.).

Homology modeling. The coordinates for the three-dimensional structure of depressant insect-selective toxin derived from the venom of *L. quinquestriatus hebraeus* ($LqhIT_2$) were obtained from Swiss-Model Protein Modeling Server [14–16]. The amino acid sequence of $LqhIT_2$ was submitted and the coordinates of the structure of the amino acids 4–60 were obtained as a PDB file. The structure of this molecule was visualized and plotted using the software written by Roger Sayle, RasMol Molecular PDB Visualization Software.

Protein determination. The amount of protein was determined using the bicinchoninic acid protein assay (Pierce) with bovine serum albumin as the standard.

RESULTS

Purification of $LqhIT_5$. The crude venom (50 mg protein) of *L. quinquestriatus hebraeus* was loaded onto a 10-ml cation-

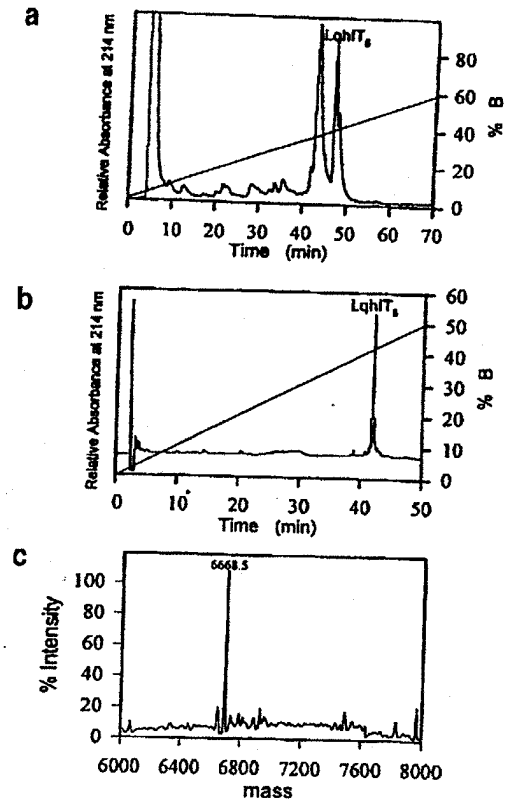


Fig. 1. Purification and mass determination of LqhIT$_5$. The purification of LqhIT$_5$ was carried out as follows. The non-binding fraction from the ion-exchange column (1.4 mg protein) was further purified by a reverse-phase C_4 column (Vydac) using HPLC (a) as described in the Experimental Procedures. The big peak that eluted from the column before LqhIT$_5$ was identified as the depressant insect toxin LqhIT$_2$ by several analytical methods such as identification with specific antibodies, electrospray ionization mass spectrometry corresponded to a mass of 6576 Da, IEF resulted in a pI value of 6.7, comobility with pure LqhIT$_2$ on reverse-phase HPLC on a Vydac C_4 column as well as symptomology when injected to blow fly larvae (data not shown). The last step of purification of LqhIT$_5$ was carried out on a microbore HPLC (Michrom Bioresources Inc.) utilizing a reverse-phase C_{18} column (b) as described in the Experimental Procedures. The molecular mass of LqhIT$_5$ (c) was determined on a VG/Fisons Quattro-BQ mass spectrometer (VG Biotech) using the maximum entropy deconvolution algorithm (MaxEnt) to transform the range *m/z* 650–1500 to give a true mass scale spectrum. Mass calibration was performed using horse heart myoglobin (Sigma Chemical Co.).

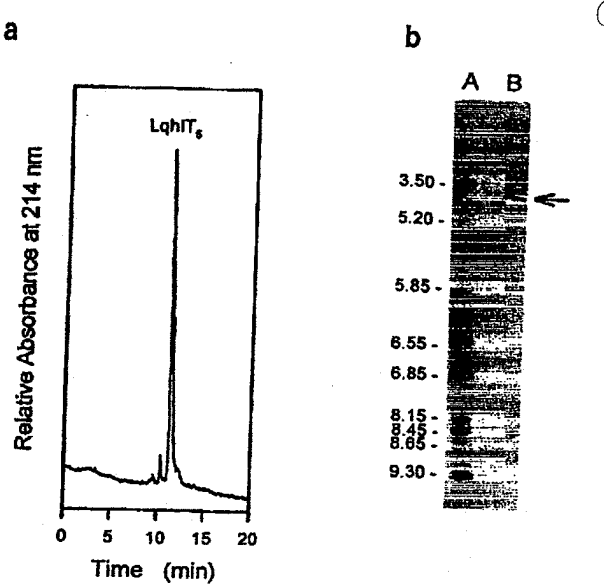


Fig. 2. Purity determination of LqhIT$_5$. The purity of LqhIT$_5$ was evaluated by free-solution capillary electrophoresis and by isoelectric focusing. Capillary electrophoresis (a). LqhIT$_5$ (0.2 mg/ml) was loaded onto a free-solution capillary column equilibrated in 20 mM sodium citrate, pH 2.9 by vacuum for 2 s. The sample was eluted from the capillary using an electric force of 20 kV and absorbance was monitored at 214 nm (a). Isoelectric focusing (b). Proteins were focused on a cast polyacrylamide gels (Ampholine PAGplate pH 3.5–9.5; Pharmacia Biotech Uppsala, Sweden). Broad-range pI markers (Pharmacia) (A) and LqhIT$_5$ (B) were loaded onto the gel and were visualized by staining with Coomassie brilliant blue G-250 [13]. The calculated pI value of LqhIT$_5$ corresponded to 3.75.

exchange column (CM-52 cellulose) and the proteins were eluted from the column. Absorbance was monitored at 280 nm and peaks collected. The toxic fraction which did not bind to the column (2 mg protein) was loaded directly on a Vydac C_4 reverse-phase HPLC column. Absorbance was monitored at 214 nm and peaks were collected accordingly (Fig. 1a). The fraction designated as LqhIT$_5$ (0.25 mg protein) was further purified on a Reliasil C_{18} column using a microbore HPLC (Fig. 1b). The single peak accounted for >97% of the absorbing material from the column and no other peak accounted for >1% of the total absorbance detected. The purity and homogeneity of LqhIT$_5$ was further evaluated using a free-solution capillary electrophoresis system which resulted in a major peak accounting for >95% of the absorbing material and showing no other significant peaks (Fig. 2a), and analytical IEF resulted in a single band (Fig. 2b). A balance study indicated that the amount of purified LqhIT$_5$ corresponded to 0.5% of the proteins in the crude venom of the scorpion. The toxicity of LqhIT$_5$ was tested by injection into blow fly larvae and mice. The amino acid sequence of LqhIT$_5$ was determined. Only a single amino acid was detected during the first cycle of Edman degradation.

Primary structure determination. The amino acid sequence of LqhIT$_5$ (Tables 1 and 2) was determined in two steps. The N-terminus (60 residues) of the reduced and alkylated toxin was determined using an HP G1000 sequence analyzer. The determination of glycine at the C-terminus was carried out utilizing electrospray ionization mass spectrometry and sequence comparison to known toxins. The major peak in the MaxEnt-transformed electrospray mass spectrum of purified LqhIT$_5$ corresponded to a molecular mass of 6668.5 Da (Fig. 1c). The result showed good agreement with the molecular mass calculated from the amino acid sequencing (6677.11 Da theoretical) assuming that four disulfide bridges are present in the native form of the peptide as it occurs in other scorpion toxins with a similar mass [17], and a glycine at the C-terminus of the toxin. This assumption is supported by the 8-Da difference between the expected mass from sequence analysis (including the glycine residue and eight thiol groups) and the mass obtained from the electrospray ionization mass spectrometry of the native form of LqhIT$_5$ (with four disulfide bonds). It also is supported by the difference between the expected pI (4.39) from the amino acid composition (using Prosis analysis) and the observed pI (3.75) on analytical IEF (Fig. 2b) as anticipated from four disulfide bonds.

Toxicity of LqhIT$_5$. LqhIT$_5$ did not show any toxicity or noticeable symptoms when injected either subcutaneously (9 μg/20 g

Table 1. Comparison of scorpion toxin amino acid sequences. Amino acids thought to confer depressant symptoms are shown in bold. BotIT$_5$, depressant insect-selective toxin derived from the venom of *Buthus occitanus tunetanus*.

Toxin		Amino acid position							
		1	10	20	30	40	50	60	70
Depressant toxins	LqhIT$_2$	DGYIKRRDGC	**KVACLIGNEG**	CDKECKAYGG	SYGYCWTWGL	ACWCEGLPDD	KTWKSETNTC	G	
	LqqIT$_3$	DGYIRKRDGC	**KLSCLFGNEG**	CNKLCKSYGG	SYGYCWTWGL	ACWCEGLPDE	KTWKSETNTC	G	
	BaIT$_2$	DGYIRRRDGC	**KVSCLFGNEG**	CDKECKAYGG	SYGYCWTWGL	ACWCEGLPDD	KTWKSETNTC	G	
	BotIT$_5$	DGYIRKRDGC	**KVSCLFGNEG**	CDKECKAYGG	SYGYCWTWGL	ACWCEGLPDD	KTWKSETNTC	G	
Depressant toxin analogs	BotIT$_2$	DGYIKGYKGC	KITCVINDDY	CDTECKAEGG	TYGYCWKWGL	ACWCEDLPED	KRWKPETNTC		
	AaIT$_5$	DGYIKRHDGC	KVTCLINDNY	CDTECKREGG	SYGYCYSVGF	ACWCEGLPDD	KAWKSETNTC	D	
	LqhIT$_5$	DGYIRGGDGC	KVSCVIDHVF	CDNECKAAGG	SYGYCWGWGL	ACWCEGLPAD	REWKYETNTC	G	
Excitatory toxins	AaIT	KKNGYAVDSSG	KAPECLLSNY	CNNQCTKVHYA	DKGYCCL-L	SCYCFGLNDDK	KVLEISDTRKSYCDT	TIIN	
	LqqIT$_1$	KKNGYAVDSSG	KAPECLLSNY	CYNECTKVHYA	DKGYCCL-L	SCYCVGLSDDK	KVLEISDARKKYCDF	VTIN	

Table 2. Similarity of scorpion toxins. The similarity was calculated using the computer software Hibio Prosis™ (Hitachi Software Engineering Co. Ltd) which compares identity between pairs of amino acids. BotIT$_5$, depressant insect-selective toxin derived from the venom of *B. occitanus tunetanus*.

Toxin	Similarity of								
	LqhIT$_2$	LqqIT$_2$	BaIT$_2$	BotIt$_5$	LqhIT$_5$	BotIT$_2$	AaIT$_5$	AaIT	LqqIT$_1$
	%								
LqhIT$_2$	100	85	95	95	74	69	75	36	34
LqqIT$_2$		100	90	91	66	60	60	35	32
BaIT$_2$			100	98	72	66	72	35	34
BotIT$_5$				100	72	66	73	35	34
LqhIT$_5$					100	69	66	31	32
BotIT$_2$						100	72	30	32
AaIT$_5$							100	34	34
AaIT								100	87
LqqIT$_1$									100

Table 3. Toxicity towards larvae of blow fly (*S. falculata*). Data for LqhIT$_2$ are from [5].

Toxin	CPU	FPU
	ng/100 ml larvae	
AaIT	2.5	—[a]
LqhIT$_2$	50	50
LqhIT$_5$	7.0	—[a]

[a] Flaccid paralysis was not detected when a high doses of 0.5 mg toxin or serial dilutions down to 1 ng were injected into 100 mg larvae.

mouse) or intracranially (4 μg/20 g mouse) into mice. LqhIT$_5$ had an excitatory effect with high toxicity when injected into blow fly larvae (7 ng/100 mg larvae). This toxicity is comparable to the toxicity of the excitatory insect toxin AaIT (Table 3) and, unlike the depressant toxin LqhIT$_2$, LqhIT$_5$ did not cause a flaccid paralysis when injected into blow fly larvae (Table 3) 5 min post injection.

DISCUSSION

The present study reports the purification and characterization of a new insect-selective excitatory toxin. This toxin (LqhIT$_5$), derived from the venom of the Israeli yellow scorpion *L. quinqestriatus hebraeus*, showed a high sequence similarity to the known depressant toxins (75% identity to LqhIT$_2$). However, unlike the depressant insect-selective toxins, LqhIT$_5$ elicited classical excitatory paralysis when injected into insects with no flaccid paralysis detected upon its injection. LqhIT$_5$ is an acidic protein with pI value of 3.75, that is highly potent toward insects (7 ng/100 mg larvae of blow fly) but shows no toxicity when injected into mice (4 μg/20 g mouse by intracranial injection).

Previous studies revealed that insect-selective toxins can be divided into two categories, the excitatory and the depressant toxins. Those toxins were shown to share a high degree of sequence similarity within each category (i.e. excitatory toxins are 80–90% identical and depressant toxins are 80–90% identical). However, a low amount of similarity exists (30–35% identity) between the excitatory and the depressant toxins. Recently, Nakagawa et al. [10] characterized a depressant toxin analog by sequence (AaIT$_5$) with no depressant or excitatory activity and a very low toxicity toward larvae of blow fly. In addition, Borchani et al. [9] reported purification of a new toxin from the venom of *Buthus occitanus tunetanus* (BotIT$_2$). The amino acid sequence of this toxin shows high similarity to the amino acid sequence of the depressant toxins. However, due to the fact that this toxin demonstrated an excitatory activity in electrophysiological studies and mammalian toxicity upon injection into mice, BotIT$_2$ was not characterized as an insect selective toxin. BotIT$_2$ as well as the depressant insect toxins were shown to affect the sodium channels in electrophysiological studies [5, 8, 9].

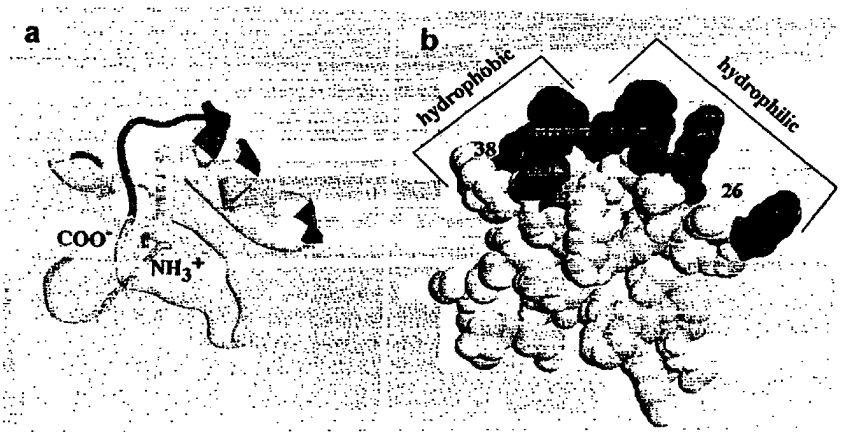


Fig. 3. Three-dimensional structure of the amino acids located at positions 4–60 of LqhIT$_2$. The amino acid sequence of the toxin LqhIT$_2$ was submitted to the Swiss-Model Protein Modeling Server and the coordinates for the three-dimensional structure were obtained. The molecule was visualized using the RasMol program and is displayed in the same orientation of the molecule in (a) and (b). (a) Carbon trace of the molecule which demonstrates the basic scaffold that is composed of an α helix, two β strands (plotted as arrows) and the loops that connect them. This basic scaffold of the molecule is common to all scorpion toxins, even those with different activities (see Discussion). Therefore, this tertiary structure of LqhIT$_2$ is similar to the tertiary structure of the depressant toxins analog LqhIT$_3$ which has 75% similarity to LqhIT$_2$ (Table 1). The N-terminus and C-terminus of the peptide (a) are indicated by NH_3^+ and COO^-, respectively. (b) Predicted orientation of the amino acids that distinguish between the depressant toxins (such as LqhIT$_2$) and the depressant toxin analogs (such as LqhIT$_3$). These amino acids are thought to be important to the depressant activity of the flaccid paralysis of the toxin (in dark gray and numbered corresponding to the sequence of the toxin in Table 1, bold) are exposed to a certain orientation of the environment and might affect a specific location on the insect sodium channel causing the flaccid paralysis effect.

The binding of the long chain scorpion toxins to the sodium channels was shown to involve multi-site attachments. The insect-selective toxins bind in close proximity on the sodium channels [18] and the depressant toxins are able to displace the excitatory toxins from their binding sites [19]. However, the depressant toxins do not displace the α insect toxins from their target sites on the sodium channels [20]. Interestingly, analysis of the three-dimensional structure of scorpion toxins affecting different target sites and having diverse mode of action (AaH$_{II}$ [21], Var3 [22], AaIT [23], charybdotoxin [24], PO5-NH$_2$ [25] and chlorotoxin [26]) reveal that they share the same basic scaffolding in the molecular structure. The toxins are compact molecules with a defined core consisting of an α helix, three anti-parallel β strands, and several loops connecting them. Scorpion toxins with very high sequence identity (95%) and structure similarities can show a different insect/mammalian toxicity ratio. For example, α insect toxin derived from the venom of *L. quinquestriatus hebraeus* (LqhαIT) [27] differs in three amino acids from α mammalian toxins derived from the venom of *L. quinquestriatus quinquestriatus* (LqqIII) [28] and is a more potent insect toxin than the latter. In addition, the toxin derived from the venom of *L. quinquestriatus quinquestriatus* (LqhIV) differs in two amino acids from LqqIV [29] and has a higher insect toxicity than LqqIV (Herrmann, R., unpublished results).

Sequence comparison of depressant and depressant-analog scorpion toxins shows a high degree of similarity among the amino acids located at the C-terminus of the toxins. However, there are some clear differences with those amino acids thought to confer depressant symptoms (Tables 1 and 2). These amino acids include the arginine residue at position 7, lysine at position 23, tyrosine at position 28, thyreonine at position 37, threonine at position 52 and a sequence of amino acids that are facing the same orientation in the tertiary structure of the molecule and include the amino acids located at residues 12−20, the lysine residue at position 23, the tyrosine residue at position 28 and the threonine residue in positions 37. These residues are different in the depressant analog group of toxins. A homology model, based on the solution NMR structures of Var3 [22] and Var1 [30], of a typical depressant toxin (LqhIT$_2$) has a variable region at residues 12-20 and several other key amino acids though to result in flaccid paralysis (Fig. 3a and b). Moreover, these amino acids demonstrate a unique hydrophobicity pattern in the tertiary structure of the molecule with a strict discrepancy between a highly hydrophobic region (amino acids 12−17, 37 and Trp38) and a highly hydrophilic region (amino acids 18, 19, 23, 28 and the lysine residue at position 26). The mammalian toxicity of BqtIT$_2$ can be related to the lysine residued located in positions 8 and 37 and the arginine residue at position 53, which differ from all other depressant and depressant-analog insect toxins.

The results of this study and the structural information described above suggest that the core structure of the scorpion toxin molecules that include the α helix and the three β strands serve as a scaffold to anchor specific amino acids at the correct orientation in three-dimensional space (Fig. 3). These amino acids and not the length of the chain appear to determine the insect or mammalian selectivities and whether the mode of action of the toxin will be excitatory or depressant. Our results with toxins from *L. quinquestriatus* caution that minor changes in specific regions of the toxin can lead to major changes in pharmacology so that groups of toxins based on length or apparent identity in sequence may not necessarily reflect the biological effects of the toxins.

Insect-selective toxins are used as pharmacological tools for the characterization of the insect sodium channel and as potency enhancing factors when cloned into baculoviruses (as insect-selective biocontrol agents). LqhIT$_3$ could serve as a tool for the clarification and identification of structures responsible for the animal group selectivity and the mode of action of scorpion toxins. The comparison of changes of the three-dimensional structure of the depressant and depressant-analog toxins could explain the selectivity of the toxins (when compared to BotIT$_2$) and the depressant or excitatory mode of action of the toxins. The identification of structures or sequences responsible for in-

sect selectivity may lead to the rational design of highly selective synthetic insecticides.

This work was supported in part by USDA Competitive Research Grants Program, 94-37302-0567; US/Israel BARD/USDA #IS-2530-95C; UC Systemwide Biotechnology Research and Education Program and E. I DuPont De Nemours & Co. U. C. Davis is a NIEHS Center of Environmental Health (P 30 ES05707) and a EPA Center for Ecological Helth Research (CR819658).

REFERENCES

1. Carbone, E., Wanke, E., Prestipino, G., Possani, L. D. & Maelicke, A. (1982) Selective blockage of voltage-dependent K^+ channels by a novel scorpion toxin, *Nature 296*, 90–91.
2. Miller, C., Moczydlowski, E., Latorre, R. & Philips, M. (1985) Charybdotoxin, a protein inhibitor of single Ca^{2+}-activated K^+ channels from mammalian skeletal muscle, *Nature 313*, 316–318.
3. Darbon, H., Zlotkin, E., Kopeyan, C., Van Rietschoten, J. & Rochat, H. (1982) Covalent structure of the insect toxin of the North African scorpion *Androctonus australis* Hector, *Int. J. Pept. Prot. Res. 20*, 320–330.
4. Rochat, H., Bernard, P. & Couraud, F. (1979) Scorpion toxins: chemistry and mode of action, *Adv. Cytopharmacol. 3*, 325–334.
5. Zlotkin, E., Eitan, M., Bindokas, V. P., Adams, M. E., Moyer, M., Burkhart, W. & Fowler, E. (1991) Functional duality and structural uniqueness of depressant insect-selective neurotoxins, *Biochemistry 30*, 4814–4821.
6. Couraud, F. & Jover, E. (1984) *Mechanism of action of scorpion toxins*, vol. 2, Marcel Dekker, New York.
7. Pelhate, M. & Zlotkin, E. (1982) Actions of insect toxin and other toxins derived from the venom of the scorpion *Androctonus australis* on isolated giant axons of the cockroach (Periplaneta americana), *J. Exp. Biol. 97*, 67–77.
8. Zlotkin, E., Kadouri, D., Gordon, D., Pelhate, M., Martin, M. F. & Rochat, H. (1985) An excitatory and a depressant insect toxin from scorpion venom both affect sodium conductance and possess a common binding site, *Arch. Biochem. Biophys. 240*, 877–887.
9. Borchani, L., Mansuelle, P., Stankiewicz, M., Grolleau, F., Cestele, S., Karoui, H., Lapied, B., Rochat, H., Pelhate, M. & El Ayeb, M. (1996) A new scorpion venom toxin paralytic to insects that affects Na^+ channel activation. Purification, structure, antigenicity and mode of action, *Eur. J. Biochem. 241*, 525–532.
10. Nakagawa, Y., Lee, Y., Lehmberg, E., Herrmann, R., Herrmann, R., Moskowitz, H., Jones, A. D. & Hammock, B. D. (1997) New anti-insect toxin (AaIT5) from *Androctonus australis, Eur. J. Biochem. 246*, 496–501.
11. Zlotkin, E., Fraenkel, G., Miranda, F. & Lissitzky, S. (1971) The effect of scorpion venom on blowfly larvae – a new method for the evaluation of scorpion venoms potency, *Toxicon 9*, 1–8.
12. Reed, L. J. & Muench, S. (1938) A simple method of estimating fifty percent end point, *Am. J. Hyg. 27*, 493–497.
13. Righetti, P. G. & Chillemi, F. (1978) Isoelectricfocusing of peptides, *J. Chromatogr. 157*, 243–251.
14. Peitsch, M. C. (1995) Protein modeling by E-Mail, *Biotechnology 13*, 723–723.
15. Peitsch, M. C. (1996) ProMod and Swiss-Model: Internet-based for automated comparative protein modelling, *Biochem. Soc. Trans. 24*, 274–279.
16. Peitsch, M. C. & Jongeneel, C. V. (1993) A 3-D model for the Cd40 ligand predicts that it is a compact trimer similar to the tumor necrosis factors, *Int. Immunol. 5*, 233–238.
17. Possani, L. D. (1984) Structure of scorpion toxins, in *Handbook of natural toxins* (Tu, A., ed.) pp. 513–550, M. Dekker, New York.
18. Gordon, D., Moskowitz, H., Eitan, M., Warner, C., Catterall, W. A. & Zlotkin, E. (1992) Localization of receptor sites for insect-selective toxins on sodium channels by site-directed antibodies, *Biochemistry 31*, 7622–7628.
19. Moskowitz, H., Herrmann, R., Zlotkin, E. & Gordon, D. (1994) Variability among insect sodium channels revealed by selective neurotoxins, *Insect. Biochem. Molec. Biol. 24*, 13–19.
20. Gordon, D. & Zlotkin, E. (1993) Binding of an alpha scorpion toxin to insect sodium channels is not dependent on membrane potential, *FEBS Lett. 315*, 125–128.
21. Housset, D., Habersetzer-Rochat, C., Astier, J. P. & Fontecilla-Camps, J. C. (1994) Crystal structure of toxin II from the scorpion *Androctonus australis* Hector refined at 1.3 A resolution, *J. Mol. Biol. 238*, 88–103.
22. Zhao, B., Carson, M., Ealick, S. E. & Bugg, C. E. (1992)) Structure of scorpion toxin Variant-3 at 1.2 A resolution, *J. Mol. Biol. 227*, 239–252.
23. Darbon, H., Weber, C. & Braun, W. (1991) Two-dimensional 1H nuclear magnetic resonance study of AaH IT, an anti-insect toxin from the scorpion *Androctonus australis* Hector. Sequential resonance assignments and folding of the polypeptide chain, *Biochemistry 30*, 1836–1845.
24. Bontems, F., Roumestand, C., Gilquin, B., Menez, A. & Toma, F. (1991) Refined structure of Charybdotoxin: common motifs in scorpion toxins and insect defensins, *Science 254*, 1521–1523.
25. Meunier, S., Bernassau, J. M., Sabatier, J. M., Martin-Eauclaire, M. F., Van Rietschoten, J., Cambillau, C. & Darbon, H. (1993) Solution structure of P05-NH2, a scorpion toxin analog with high affinity for the apamin-sensitive potassium channel, *Biochemistry 32*, 11969–11976.
26. Lippens, G., Najib, J., Wodak, S. J. & Tartar, A. (1995) NMR sequential assignments and solution structure of Chlorotoxin, a small scorpion toxin that blocks chloride channels *Biochemistry 34*, 13–21.
27. Eitan, M., Fowler, E., Herrmann, R., Duval, A., Pelhate, M. & Zlotkin, E. (1990) A scorpion venom neurotoxin paralytic to insects that affects sodium current inactivation – purification, primary structure, and mode of action, *Biochemistry 29*, 5941–5947.
28. Kopeyan, C., Mansuelle, P., Martin-Eauclaire, M. F., Rochat, H. & Miranda, F. (1993) Characterization of toxin III of the scorpion *Leiurus quinquestriatus quinquestriatus*: a new type of alpha-toxin highly toxic both to mammals and insects, *Natural Toxins 1*, 308–312.
29. Kopeyan, C., Martinez, G. & Rochat, H. (1985) Primary structure of toxin IV of *Leiurus quinqestriatus quinqestriatus*. Characterization of a new group of scorpion toxins, *FEBS Lett. 181*, 211–217.
30. Lee, W., Jablonsky, M. J., Watt, D. & Krishna, N. R. (1994) Proton Nuclear Magnetic Resonance geometry/simulated annealing studies on the Variant-1 neurotoxin from the new world scorpion *Centruroides sculpturatus Ewing, Biochemistry 33*, 2468–2475.
31. Cestele, S., Kopeyan, C., Oughideni, R., Mansuelle, P., Granier, C. & Rochat, H. (1997) Biochemical and pharmacological characterization of a depressant insect toxin from the venom of the scorpion *Buthacus arenicola, Eur. J. Biochem. 243*, 93–99.
32. Borchani, L., Stankiewicz, M., Kopeyan, C., Mansuelle, P., Kharrat, R., Cestele, S., Karoui, H., Rochat, H., Pelhate, M. & Ayeb, M. E. (1997) Purification, structure and activity of three insect toxins from *Buthus occitanus tunetanus* venom, *Toxicon 35*, 365–382.
33. Kopeyan, C., Mansuelle, P., Sampieri, F., Brando, T., Bahraoui, E. M., Rochat, H. & Granier, C. (1990) Primary structure of scorpion anti-insect toxins isolated from the venom of *Leiurus quinquestriatus quinquestriatus, FEBS Lett. 261*, 423–426.

Eur. J. Biochem. 238, 653–660 (1996)

A recombinant insect-specific α-toxin of *Buthus occitanus tunetanus* scorpion confers protection against homologous mammal toxins

Balkiss BOUHAOUALA-ZAHAR[1], Frédérique DUCANCEL[2], Ilham ZENOUAKI[1], Rym BEN KHALIFA[3], Lamia BORCHANI[1], Marcel PELHATE[3], Jean-Claude BOULAIN[2], Mohamed EL AYEB[1], André MÉNEZ[2] and Habib KAROUI[1]

[1] Laboratoire Venins et Toxines, Institut Pasteur de Tunis, Tunis, Tunisie
[2] Département d'Ingénierie et d'Etudes des Protéines, Gif-sur-Yvette, Commissariat à l'Energie Atomique, Saclay, France
[3] Laboratoire de Neurophysiologie, ERS 108, CNRS, Angers, France

(Received 21 December 1995/1 March 1996) – EJB 95 2102/1

We have constructed a cDNA library from venom glands of the scorpion *Buthus occitanus tunetanus* and cloned a DNA sequence that encodes an α-toxin. This clone was efficiently expressed in *Escherichia coli* as a fusion protein with two Ig-binding (Z) domains of protein A from *Staphylococcus aureus*. After CNBr treatment of the fusion protein and HPLC purification, we obtained approximately 1 mg recombinant α-toxin/l bacterial culture. The toxin, called Bot XIV, displays no toxicity towards mammals but is active towards insects as shown by its paralytic activity against *Blatella germanica* cockroach and by electrophysiological studies on *Periplaneta americana* cockroaches. The Bot XIV protein fused to two Z domains is highly immunogenic in mice and induces production of antisera that specifically recognize and neutralize highly toxic components that had been injected into mice. This fusion protein could be very useful for development of potent protective antisera against scorpion venoms.

Keywords: scorpion α-toxin; cDNA; expression in *Escherichia coli*.

Scorpions and their venoms have attracted the attention of many investigators, mainly because they constitute a hazard to human life and health. In Tunisia, two dangerous scorpion species (*Androctonus australis hector* and *Buthus occitanus tunetanus*) are a public health problem. Development of efficient and selective protections against these venoms could be of great clinical importance. Serotherapy is the most common approach to protect populations against scorpion venoms. However, the availability of potent and reliable anti-venom sera is limited. Within a given venomous species, venom is composed of a wide variety of components which may be toxic or non-toxic to mammals. Moreover, the relative proportions of toxic and non-toxic components varies between scorpion venoms (El Ayeb and Rochat, 1985).

The toxicity of scorpion venoms is mainly due to small, basic, single-chain proteins of 60–65 amino acid residues (Miranda et al., 1970) whose structure is tightly restricted by four disulfide bridges (Gregoire and Rochat, 1983). These proteins bind with high affinity to specific sites in excitable tissues (Catterall, 1986), and modify sodium-channel conductance (reviewed by Hille, 1992). *Buthidae* scorpion venoms contain different toxins that are selectively active on mammal and/or insect sodium channels (Gordon et al., 1984), which have been classified mainly as α-type (Rochat et al., 1979; Catterall, 1984) or and β-type toxins (Jover et al., 1980; Couraud et al., 1982; De Lima et al., 1986), based on their binding, pharmacological and electrophysiological properties.

Four pharmacological groups of toxins have been characterized. The first group includes α-toxins, which affect mammals and/or insects through extreme prolongation of the sodium-channel inactivation (Catterall et al., 1976; Rochat et al., 1979; Gordon and Zlotkin, 1993). The second group consists of β-toxins, which affect sodium-channel activation and bind in a potential-independent manner (Couraud et al., 1982). The third group includes depressant insect-selective toxins, which induce progressive flaccid paralysis of insects (Lester et al., 1982; Zlotkin et al., 1985, 1991; Kopeyan et al., 1990). The fourth group contains the excitatory insect-selective toxins, which cause rapid and sustained spastic contractive paralysis with induction of repetitive channel action (Zlotkin et al., 1971 a, b; Walther et al., 1976; Pelhate and Zlotkin, 1981, 1982).

Toxins from *Buthidae* venoms constitute a family of closely related peptides, which could be classified into several structural groups depending on their amino acid sequences (Rochat et al., 1979). Immunological data have shown that the antigenic groups are the same as the structural groups (Delori et al., 1981). Moreover antitoxins only recognize and neutralize toxins that belong to the same group (Delori et al., 1981; El Ayeb et al., 1983a). There is no overlap between antigenic regions and the pharmacologically active site of scorpion toxins (El Ayeb et al., 1986).

Despite the structural, antigenic, pharmacological and electrophysiological diversity, the overall tertiary structure of scorpion toxins shows a common organizational feature that is characterized by a small triple-stranded antiparallel β-sheet linked to a short α-helix by two disulfide bridges, and to an extended fragment by one disulfide bridge (Fontecilla-Camps et al., 1980, 1988; Darbon et al., 1991).

Thirteen toxins have been purified and characterized from *B. occitanus tunetanus* venom (Miranda et al., 1970). They are active on mammals and belong to two structural and antigenic groups (Rochat et al., 1979; El Ayeb et al., 1983a). The most active toxin, BotIII, is antigenically related to toxin II of *A. australis hector*. The remaining majority of *B. occitanus tuneta-*

Correspondence to B. Bouhaouala-Zahar, Laboratoire Venins et Toxines, Institut Pasteur de Tunis, 13 place Pasteur, 1002 Bélvédère, BP 74, Tunis, Tunisia

Abbreviation. LD_{50}, median lethal dose.

nus toxins form another structural and antigenic group and do not include toxins from *A. australis hector* venom. Each toxin that belongs to this group BotI and BotII from *B. occitanus tunetanus* (Miranda et al., 1970), BomIII from *Buthus occcitanus mardochei* (Vargas et al., 1987), toxin III from *Leiurus quinquestriatus quinquestriatus* (Kopeyan et al., 1993), LqhαIT from *Leiurus quinquestriatus hebraeus* (Eitan et al., 1990)] shows varying toxicity towards mammals and/or insects. Toxins that belong to two other structural and antigenic groups of toxins from *A. australis hector* (AaHI, AaHI′, AaHII, AaHIII) have been cloned, sequenced and expressed (Bougis et al., 1989).

We therefore decided to construct and screen a cDNA library from scorpion glands with probes specific to conserved regions of toxins related structurally and antigenically to BotI (Bouhaouala-Zahar et al., 1996). We produced a recombinant protein, studied its pharmacological and electrophysiological properties and its capacity to trigger production of antibodies that could cross-react with BotI-related group toxins. We show that this approach was successful and that it may aid development of immune-prevention strategies and serotherapy development against scorpion venoms.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* DH5α[*F′Φ80Δ* (*lacZ*)M15 *Δ*(*lacZYAargF*) U169 *recA1 endA1 hsdR17* (r_k^-,m_k^+) *supE44 thi-1 gyrA96 relA1*] (Hanahan, 1983) was used as bacterial host for cloning. Expression of recombinant toxin (fused to two Z domains) was performed in *E. coli* strain HB101 [*F⁻Δ*(*mcrC-mrr*) *leu supE44 ara14 galK2 lacY1 proA2 rpsL20* (*Str*^{r}) *xyl-5 mtl-1 recA13*] (Boyer and Roulland-Dussoix, 1969). Plasmids pUC18, pCDV-1 (vector for cDNA cloning) and pEZZ 18 (protein-A−gene fusion vector) were obtained from Pharmacia.

Purification of telson mRNA. 80 *B. occitanus tunetanus* venom glands were used to purify mRNA. Telsons were removed 2 d after manual extraction of their venom, to allow the toxin-producing cells of the venom glands to enter into the secretory phase. Tissues of telsons were homogenized and total RNA extracted by means of the guanidium/hot-phenol method (Maniatis et al., 1982). Poly(A)-rich RNA was purified on an oligo(dT)-cellulose spun column (Pharmacia).

Construction and screening of the cDNA library. Synthesis of cDNA was performed by means of the Okayama-Berg cloning strategy (Okayama and Berg, 1983). 5 µg telson mRNA was hybridized with oligo(dT)-labeled pCDV-1 to prime first-strand cDNA synthesis. First-strand synthesis was achieved by means of avian myeloblastosis virus reverse transcriptase. Synthesis of the second strand of the cDNA was performed in the presence of *E. coli* RNase H, DNA polymerase I and T4 DNA ligase. Resulting recombinant plasmids were used to transform competent *E. coli* DH5α cells, with an efficiency of transformation of 4×10^7 clones/µg recombinant pCDV-1. The library was screened according to the procedure described by Woods (1984) with two ^{32}P-labeled probes specific for the N-terminal and C-terminal conserved regions of toxin I, and toxin II of *B. occitanus tunetanus*:

probe 1

Glu10 Asn Cys Val Tyr Glu Cys16

5′ GA^A_G AA^T_C TG^T_C GTI TA^T_C GA^A_G TG^T_C GC 3′;

probe 2

Tyr42 Gly Asn Ala Cys Trp Cys (Lys, Ile) Asp50

5′ TA^T_C GGI AA^T_C GCI TG^T_C TGG TG^T_C ^T_AAI ^A_GA 3′.

Probes were purchased from *Unité de Chimie organique* (Institut Pasteur de Paris) and were labeled by means of T_4 polynucleotide kinase. Filters were prehybridized for 2 h at 37°C in 6×NaCl/Cit (NaCl/Cit is 0.15 M NaCl, 0.015 M sodium citrate, pH 7), 1×Denhardt's, 0.05%, 0.5% SDS and 100 µg/ml sheared, denatured herring sperm DNA. Filters were hybridized for 16 h at 37°C in 6×NaCl/Cit, 1×Denhardt's, 0.05% $NaPP_i$, 20 µg/ml yeast tRNA and ^{32}P-labeled oligonucleotide probes. 26 specific clones were selected for further analysis. Size characterization of cDNA inserts was performed by means of restriction enzymes *Xho*I and *Rsa*I. After orientated subcloning into pUC18 and pUC19 vectors, cDNA inserts were completely sequenced in both directions by the dideoxynucleotide sequencing method (Sanger, 1977).

Production and purification of the fusion protein. *E. coli* HB101 transformed with the expression vector was grown in a 5-l fermentor (LSL Biolafitte) in 4 l tryptic soy broth medium (Difco) supplemented with 5 g/l glucose and 200 µg/ml ampicillin. The temperature was 37°C, the pH was maintained at 7.2 with 5 M NaOH, and the oxygen concentration was adjusted to 80% saturation by addition of an increasing proportion of pure oxygen to the air supply. When the glucose concentration fell to 0.5 g/l, feeding was initiated by addition of glucose (40%) as carbon source. The feeding rate was adjusted to give a growth rate of 0.3 h^{-1}. When the culture reached early stationary phase, cells were precipitated by centrifugation, and the supernatant was filtered through a 0.2-µm filter and concentrated by ultrafiltration on a membrane with a molecular mass cut-off of 10 kDa (Filtron). The solution was purified by affinity chromatography on an IgG-Sepharose column as described by Ducancel et al. (1989) and lyophilized.

Cleavage and purification of the recombinant toxin. The procedure followed to cleave the fusion protein by CNBr treatment was that described by Boyot et al. (1990). Lyophilized fusion protein was dissolved in 0.1 M HCl in the presence of a 500-fold molar excess of CNBr at room temperature for 24 h. Purification of the resulting recombinant toxin was performed by chromatography on a reverse-phase HPLC column (Vydac, C_3) equilibrated in 0.1% trifluoroacetic acid, followed by trifluoroacetic acid/CH_3CN/H_2O elution.

Characterization of the fusion protein. Visualization of the recombinant toxin was performed by SDS/PAGE (15% acrylamide) on the PhastSystem (Pharmacia). The affinities of the fusion protein to antibodies directed against BotG-50 (a partially purified mixture of *B. occitanus tunetanus* toxins), BotI and AaHII toxins were determined by means of ELISA methods described by Chavez-Olortegui et al. (1991). Immunoblot analysis was performed by electroblotting of proteins after SDS/PAGE onto poly(vinylidene difluoride) filters, then treated with total rabbit antisera raised against native venom toxins. Median lethal doses (LD_{50}) were determined by intra-cerebroventricular injections of purified recombinant protein in 20-g C_{57} Black/6 mice.

Electrophysiological techniques. Voltage-clamp and current-clamp assays were performed on isolated giant axons dissected from abdominal nerve cords of adult male cockroaches *Periplaneta americana* by means of the double-oil-gap-and-the-single-fiber technique (Pichon and Boistel, 1967; Pelhate and Sattelle, 1982) at 18−20°C.

Hybrid proteins, BotXIVr1 and BotXIVr2 were dissolved to 1.4×10^{-6} M in 200 mM NaCl, 3.1 mM KCl, 5.4 mM $CaCl_2$, 5.0 mM $MgCl_2$, 1 mM Hepes, pH 7.0 (buffer A), then externally applied to the axonal membrane.

To selectively block (or largely decrease) the potassium current, 0.5 mM 3,4-diaminopyridine was used (Pelhate and Pichon, 1974).

Immunization procedure and ELISA. C57 Black/6 mice (6–8) were immunized with IgG-purified recombinant BotXIV proteins (fused to two Z domains) as immunogen. 50 µg immunogen were emulsified in complete Freund's adjuvant and injected by intraperitoneal and subcutaneous routes on day 1. Each mouse was injected with 100 µg, 150 µg and 200 µg immunogen in incomplete Freund's adjuvant on days 8, 17 and 35, respectively. 42 d after the initial injection, mice were bled from the retro-orbital sinus.

ELISA were used to assess cross-antigenicity of purified recombinant IgG-purified BotXIVr with BotG-50, AahG-50 and BotI toxins. Optimization of the previously described procedure (Chavez-Olortegui et al., 1991) was performed. Microtitration plates were coated with 5 µg/ml toxin in 0.1 M sodium bicarbonate, pH 9.6, for 90 min at 37°C. After washing for five times with 150 mM NaCl, 50 mM sodium phosphate (NaCl/P_i) containing 0.05% Tween-20 (NaCl/P_i/Tween-20), non-specific sites were saturated with NaCl/P_i containing 5% BSA for 1 h at 37°C and the plates washed three times with NaCl/P_i/Tween-20. Appropriate dilutions of sera in NaCl/P_i/Tween-20 were added and incubated for 90 min at 37°C, then for 15 min at 4°C. After washing with NaCl/P_i/Tween-20, 100 µl peroxidase-conjugated anti-mouse Ig (diluted 1000-fold) was added to each well and incubated at 37°C for 90 min and 4°C for 15 min. 200 µl 0.4 mg/ml *O*-phenylenediamine in sodium citrate, pH 5.2, 0.03% H_2O_2 were added to each well and the plates incubated in the dark for 5 min at room temperature. The reaction was stopped by addition of 50 µl 1 M sulfuric acid. Absorbance at 492 nm was measured in a microplate reader (Titertek Multiskan Photometer MCC/340).

Neutralizing capacity of immune sera. The neutralizing capacity of immune sera was evaluated after subcutaneous injection. Samples of toxic fractions were incubated with 150 µl immune sera in 200 µl, for 90 min at 37°C and for 120 min at 4°C, and injected into 20-g naïve Swiss mice. The LD_{50} of BotG-50 was determined after incubation with immune sera and the neutralizing activity was calculated as recommended by the World Health Organization (1981) as follows:

Neutralizing capacity = LD_{50} (in presence of immune sera)−LD_{50} (in absence of immune sera)/volume of immune sera.

***In vivo* protection assay.** One month after the end of immunization program, groups of 4–6 immunized mice were injected subcutaneously with increasing amounts of BotG-50. Death was recorded after 24 h.

***In vivo* insect toxicity.** *In vivo* toxicity of purified BotXIV toxin was assessed on male *Blatella germanica* (50 mg body weight). Four *Blatella* were used/dose. 0.5–2 µl was injected in the abdominal segment. Toxicity was monitored by lethality test after 1 h. For all injections, the solvent used was 0.15 M NaCl, 1 mg/ml BSA. LD_{50} values were calculated according to the Reed and Muench method (1938).

RESULTS

Screening of the cDNA library. The cDNA library was constructed from 5 µg poly(A)-rich mRNA and was estimated to include 5.4×10^4 recombinant clones. After two successive screenings with probe 1 or probe 2 (see above), 26 positive clones were selected. Of these, clone 4E4 (BotXIVr) was subcloned and completely sequenced (Fig. 1).

Amino acid sequence of toxin precursor. The sequence depicted in Fig. 1 corresponds to a full-length cDNA clone. It contains 468 nucleotides [excluding the poly(A)-rich tail] with an ORF of 396 bp. At the 5′ end, multiple potential translation-initiation codons were observed (Kozak, 1984). Comparison of the amino acid sequence with those of previously described scorpion α-toxin precursors (Kozak, 1984; Bougis et al., 1989) allowed us to propose that the methionine-residue codon located at position −18 is the initiating codon. The 3′ end contains a putative polyadenylation signal, AATAAA, 18 nucleotides upstream of the poly(A)-rich tail (Proudfoot and Brownlee, 1976). We showed, by alignment with sequences of toxins from *B. occitanus tunetanus*, *A. australis hector* and *B. occitanus mardochei*, that the deduced amino acid sequence corresponds to a toxin-like sequence. The sequence presents 57% identity with BotI and BotII, 36% identity with BotIII and BotXI and approximately 70% identity with BomIII toxin of *B. occitanus mardochei* (Vargas et al., 1987). Furthermore, the N-terminal region is highly conserved, the positions of the eight cysteine residues are maintained, and the number of amino acid residues of the precursor is similar to those of other toxins from *B. occitanus tunetanus*. Thus, we termed the protein BotXIV, for *B. occitanus tunetanus* XIV recombinant toxin.

Expression and hybrid characterization. BotXIV was expressed in *E. coli* HB101, as a fusion protein with two IgG-binding (Z) domains derived from protein A of *S. aureus*. The expression vector used for this purpose was pEZZ18 (Löwenadler et al., 1987; Nilsson et al., 1987). It is a secretion vector designed for translocation of Z-domain-containing fusion proteins into the growth medium of bacteria. Expression-vector construction was performed after modification by PCR amplification of the cDNA sequence that encodes the precursor sequence of the toxin. The sense oligonucleotide (5′ GGGAGCGGAGGGTACCCATGGTACGTGACGGTTATATT 3′) introduced a *Kpn*I restriction site (underlined), and a methionine residue (bold) at position −1. Since the mature sequence of BotXIV has no methionine residue, it constitutes a unique and useful CNBr-cleavage site, as previously described for other hybrid snake toxins (Ducancel et al., 1989; Boyot et al., 1990; Hodgson et al., 1993; Danse et al., 1994). The antisense oligonucleotide (5′GGGAGCGGCAGGATCCTTATCAGCGATGGCATTTT 3′) was designed to introduce a *Bam*HI restriction site and the stop codon TAA (bold), which is efficiently recognized in *E. coli*. Thus, the construct pEZZ/BotXIVr contained an ORF encoded successively the protein A signal peptide, two IgG-binding domains (15 kDa), an eight-amino-acid linker peptide (-Ala-Asn-Ser-Ser-Ser-Val-Pro-Met-) and BotXIV (7.5 kDa) mature sequence. SDS/PAGE (15% acrylamide) analysis revealed that an IgG-affinity-purified protein with an apparent molecular mass of 24 kDa (Figs 2 and 3) was present in the culture medium and was weakly degraded, as demonstrated by the presence of very few low-molecular-mass proteins (Fig. 2). We estimated the yield of recombinant BotXIV toxin as approximately 18 mg/l bacterial culture. The biological properties of the fusion protein towards mammals and insects revealed that 2.5 µg fusion protein injected intra-cerebroventricularly into mice did not show any toxic effect, and that no excitatory activity was detected on the giant axons of *P. americana* (data not shown).

Purification and properties of BotXIV. IgG-purified fusion protein was cleaved by CNBr treatment as previously described (Boyot et al., 1990). Over 24 h, the major fusion protein (24 kDa) was gradually split into two products: the ZZ domains and a protein of approximately 7 kDa (Fig. 3). BotXIV was further purified by HPLC chromatography on a C_3 column and was recovered as two peaks named BotXIV1 (33.3%) and BotXIV2 (66.6%) (data not shown). Chemical characterization (data not

```
5'TACGTGAAAGTGCCGGACACATTGGATGTATGGTTTGACTCCGGATCTACCCACTCTTCTGTTGT
TGACGTGCGTCCGGAATTTGCCGGTCACGCAGCGGACATGTATCTGGAAGGTTCTGACCAACACCGCGGCTGGTTC

ATG TCT TCC CTA ATG ATC TCC ACC GCG ATG AAG GGT AAA GCG CCG TAT CGT CAG GTA
 M  S  S  L  M  I  S  T  A  M  K  G  K  A  P  Y  R  Q  V

CGT GAC GGT TAT ATT GCC CAG CCC CAT AAC TGT GCA TAC CAT TGT TTA AAA ATC TCC
 R  D  G  Y  I  A  Q  P  H  N  C  A  Y  H  C  L  K  I  S

TCA GGC TGC GAC ACT TTA TGT AAG GAG AAC GGT GCT ACG AGT GGC CAC TGC GGA CAT
 S  G  C  D  T  L  C  K  E  N  G  A  T  S  G  H  C  G  H

AAA TCT GGA CAC GGA AGT GCC TGC TGG TGC AAA GAC TTG CCA GAT AAA GTA GGG ATT
 K  S  G  H  G  S  A  C  W  C  K  D  L  P  D  K  V  G  I

ATA GTA CAT GGA GAA AAA TGC CAT CGC TGA TAA ATCTGTAAAGCAAAAACCAAAGAATGTATTTT
 I  V  H  G  E  K  C  H  R  end end

AAGAACTATTAAATAAATATAAATAATAAAATTG(A)n
```

Fig. 1. Complete nucleotide sequence of the mRNA that encodes BotXIV toxin precursor. Deduced amino acid sequence is given in single-letter code. Amino acids that form the signal peptide are underlined. The putative polyadenylation site at the 3'terminus is in bold.

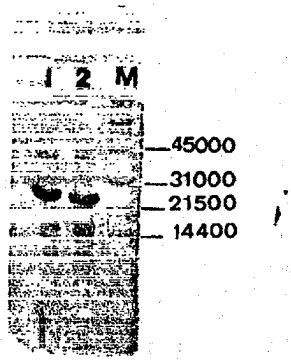


Fig. 2. SDS/PAGE (20% PhastGel) of periplasmic bacterial medium purified on an IgG-Sepharose column. Lanes 1 and 2, purified recombinant BotXIV from culture-medium fraction of Hb101 host transformed with two isolates of pEZZ/BotXIV; lane M, molecular-mass markers (Da). The culture were treated as described in Materials and Methods.

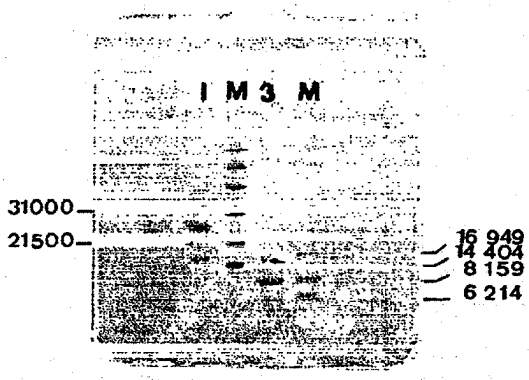


Fig. 3. SDS/PAGE (20% Phast-gel) of a cleaved BotXIV. Recombinant BotXIV (lane1) was cleaved by CNBr and purified on a HPLC column (lane 3), as described under Materials and Methods. BotXIV appears as a band of approximately 7 kDa; lanes 2 and 4, low-molecular mass and medium-molecular-mass markers (Da).

shown) revealed that both compounds have similar apparent molecular masses and pI as detected on SDS/PAGE and IEF, respectively, and identical amino acid compositions and N-terminal sequences. At least two possible explanations can account for this observation. First, the peaks correspond to two conformers of BotXIV; second, one peak represents partially unfolded product, as previously observed for recombinant α-bungarotoxin (Rosenthal et al., 1994). The final yield of purified recombinant toxin was approximately 1 mg/l of culture.

Cross antigenicity of purified BotXIV. HPLC-purified BotXIV (mixture of forms 1 and 2) was recognized in ELISA by anti-BotI serum (Fig. 4). In contrast, no cross-antigenicity was observed with anti-AahII serum. These results suggest that BotXIV belongs to the same antigenic group as BotI, which is different from that of AahII (El Ayeb et al., 1983a). In agreement with this result, BotXIV shows higher sequence similarity with BotI than with AahII.

Biological assays of BotXIV (*in vivo* toxicity of BotXIV). Intra-cerebroventricular injections in Swiss mice of purified toxins (2.5 µg BotXIV1 or BotXIV2), did not have any toxic effect. Since LD_{50} values are usually in the range 2–100 ng for highly

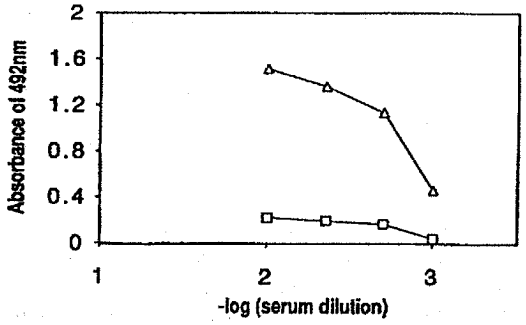


Fig. 4. Cross-antigenicity of BotXIV with BotI and AahII. Binding of anti-BotI (△) and anti-AahII (□) serum to plates coated with recombinant, HPLC-purified BotXIV are revealed with anti-rabbit Ig coupled to peroxidase by means of ELISA.

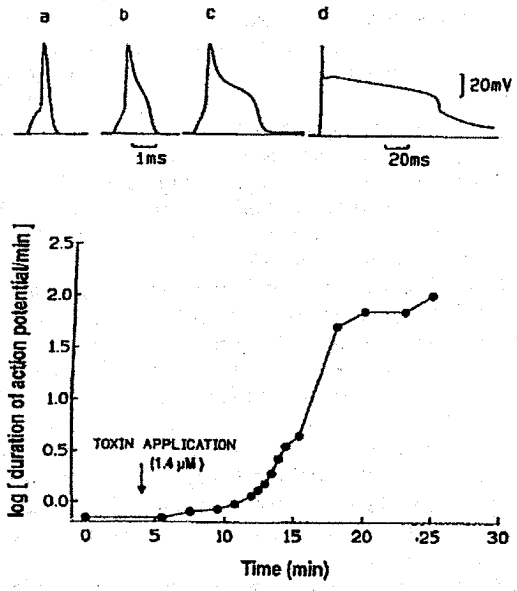


Fig. 5. **Effects of BotXIV1 (1.4 µM) on the action potential of isolated cockroach axon.** Action potentials evoked by a short (0.5 ms) depolarizing current pulse of 18 nA, 0 (a), 8 (b), 12 (c) and 20 min (d) after BotXIV1 application. Note the different horizontal scale in (d). The graph shows the evolution of the action-potential duration over time in the presence of the recombinant toxin.

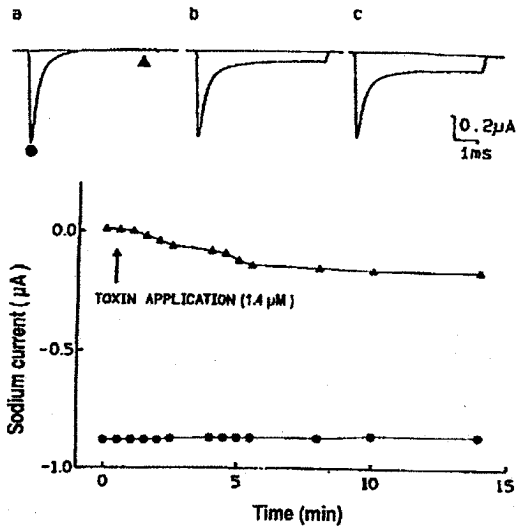


Fig. 6. Effect of BotXIV1 (1.4 µM) on the sodium current of isolated cockroach axon. Voltage-clamp experiments were performed in the presence of 0.5 mM 3,4-diaminopyridine. The membrane potential was lowered to −20 mV from a holding potential of −60 mV. The membrane sodium current recorded in normal conditions (a), and 5 min (b) and 14 min (c) after of BotXIV1 application are shown. The graph indicates the evolution of the peak (●) and the late (▲) sodium inward current upon recombinant-toxin addition.

to weakly toxic toxins, we conclude that BotXIV displays no toxicity on mammals. In contrast, when injected into *Blatella*, BotXIV induced contractive paralysis. The LD_{50} was in the range 11.2−12.4 µg/g body weight for BotXIV1 (data not shown). The LD_{50} for BotXIV2 was higher than 20 µg/g. The weaker toxicity of BotXIV2 may be due to partial folding of the protein.

Effect of BotXIV1 on cockroach axonal membrane. BotXIV1 (1.4 µM) was tested in current-clamp conditions. No effects on the amplitude of the action potential or on the level of the membrane resting potential were observed (Fig. 5). In contrast, BotXIV1 induced progressive (Fig. 5) prolongation of the evoked action potential. BotXIV1 increased the duration of the action potential over time, which leads to a plateau potential that lasted for 110 ms 14 min after toxin application (Fig. 5).

Membrane sodium currents were measured under voltage-clamp conditions in response to depolarization from a holding potential of −60 mV to a membrane potential of −20 mV, after suppression of the potassium current with 0.5 mM 3,4-diaminopyridine. Fig. 6 illustrates the evolution of peaks and late sodium inward currents. Under normal conditions, the late sodium inward current was transient and completely inactivated in less than 3 ms (Fig. 6a). After BotXIV1 application, we noted progressive development of a maintained sodium current (Fig. 6b) which was approximately 18% that of the peak current (Fig. 6c) and reached 20−25% in other experiments. BotXIV1 did not affect the peak value of the inward sodium current and at the end of the voltage pulse, the maintained current rapidly returned to zero (fast normal deactivation). In another series of assays, it was demonstrated that BotXIV1 (1.4 µM) did not modify potassium conductance (after selective suppression of late inward sodium current with 1 µM tetrodotoxin toxin).

Effect of BotXIV2 on cockroach axonal membrane. In current-clamp experiments, 1.4 µM BotXIV2 for 12 min had no effect on the membrane action potential. A limited increase in the duration of the action potential (but no plateau potential) was obtained in the presence of this isoform. In voltage-clamp assays, the maximum inward maintained sodium current corresponded to 12% that of the peak (data not shown).

Antibodies raised against BotXIV are neutralizing. We successively injected mice with 50, 100, 150 and 200 µg recombinant BotXIV (fused to two Z domains). The pooled serum from six mice was used to establish antibody titers and immunological responses (Fig. 7). The immune antisera raised against BotXIV preferentially recognize BotG-50 and BotI. AahG-50, which is known not to contain toxins antigenically related to BotI, reacts weakly with anti-BotXIV. These data support the specificity of anti-BotXIV towards BotI-antigenic-group members. Furthermore, the immune serum obtained was neutralizing, since incubation of 105.6 µg BotG-50 (fourfold higher than the LD_{50}) in the presence of the anti-hybrid serum, induced only a 50% toxic effect in mice (Table 1). Complete protection was observed

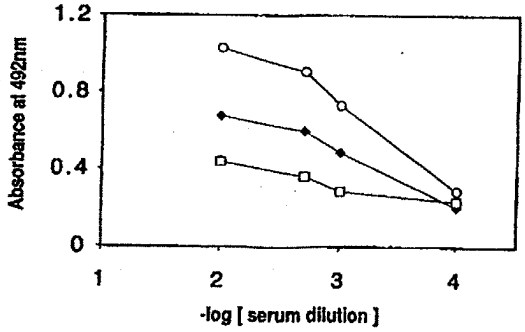


Fig. 7. Immunoreactivity of anti-BotXIV Ig. 5 μg/ml BotG-50 (○), AahG-50 (□) and BotI (◆) were adsorbed to plates and ELISA were prepared with various serum dilutions of anti-BotXIV Ig. Absorbance was recorded at 492 nm and non-specific binding substracted. The immune sera was pooled from six mice.

Table 1. Passive and active protection of mice against BotG-50 lethality. LD_{50} of BotG-50 was 1.32 μg/g. Numbers in parenthesis indicate the number of mice injected.

Bot G-50 challenge dose	Anti-BotXIV serum neutralization activity	Protection of vaccinated mice
μg	% survival	
52.8	100 (6)	100 (4)
79.2	67 (6)	83 (6)
105.6	50 (6)	50 (6)

when 52.8 μg BotG-50 were incubated with 150 μl immune serum prior to injection. Thus, partial but significant neutralization was observed when 79.2 μg and 105.6 μg BotG-50 (i.e. threefold and fourfold higher than the LD_{50}, respectively) were used. Furthermore, the neutralizing capacity of the serum was specifically due to antibodies raised against the hybrid since the preimmune serum had no neutralizing effect (data not shown). The deduced neutralizing capacity was 528 μg BotG-50/ml, or approximately 1.6 mg crude venom/ml. The LD_{50} of BotG-50 was estimated as 26.4 μg/20-g Swiss mouse.

***In vivo* protection of mice against BotG-50 lethality.** In agreement with the neutralizing potency of anti-BotXIV serum, immunized mice were significantly protected against challenge with 52.8, 79.2 and 105.6 μg BotG-50 toxic fractions (Table 1). Injection of 52.8 μg BotG-50 did not generate any toxicity sign. In contrast, injection of 23 μg AaHG-50 (twofold higher than the LD_{50}) was toxic against mice (data not shown). 50% protection was observed when the challenge dose was as high as 105.6 μg BotG-50. This value correspond to approximately 105 μg BotG-50 and 210 μg *B. occitanus tunetanus* venom. It is established that BotG-50 is approximately 50% of crude venom (Miranda et al., 1970).

These data indicated that antibodies raised against the BotXIV hybrid have neutralizing activity specifically to venoms of *B. occitanus tunetanus* species. Therefore, an efficient immune protection could be developed against the toxins of these venoms by injection of low doses of BotXIV.

DISCUSSION

From a *B. occitanus tunetanus* cDNA library, we elucidated the sequence of the precursor of BotXIV, an insect α-toxin. In the BotXIV precursor, the signal peptide is followed by a sequence that codes for a typical scorpion toxin, charaterized by the presence at conserved positions of eight cysteine residues and a conserved N-terminal sequence. The overall amino acid sequence shows more than 50% similarity with toxins that belong to the same structural and antigenic group as BotI (El Ayeb et al., 1983b). This group includes toxins active against mammals (BotI, BotII and BomIII) and against insects [LqqIII (Kopeyan et al., 1993) and LqhαIT (Eitan et al., 1990)]. BomIII (Vargas et al., 1987) presents the highest similarity (70%) with BotXIV. The system chosen to express BotXIV in *E. coli* was previously demonstrated to be suited to the production of soluble, correctly folded snake venom toxins (Ducancel et al., 1989; Hodgson et al., 1993; Pillet et al., 1993; Danse et al., 1994; Trémeau et al., 1995). This system, however, had not been used to produce recombinant scorpion toxins. The present attempt was therefore interesting because several other systems failed to generate satisfactory levels of active recombinant scorpion toxins. Expression of scorpion toxins in Cos-7 cells (Bougis et al., 1989), in insect cells by means of baculovirus system (Carbonell et al., 1988), in plants (Pang et al., 1992), in NIH/3T3 mouse cells (Dee et al., 1990) and in yeast (Pang et al., 1992; Martin-Eauclaire et al., 1994) led to rather low yields. Recently, Zilberberg et al. (1995) described the expression in bacteria of a cDNA that encoded an α anti-insect neurotoxin (LqhαIT). However, in this system the recombinant toxin accumulates as insoluble aggregates with an extra N-terminal methionine residue and thus requires denaturation/renaturation treatment to obtain a soluble product. We demonstrated that from a high expression of recombinant BotXIV (fused to two Z domains) after CNBr cleavage we obtained approximately 1 mg purified recombinant BotXIV toxin/l bacterial culture. Such an amount is sufficient to carry out appropriate biochemical and functional studies and immunization experiments.

BotXIV displayed antigenic cross-reactivity with BotI toxin and BotG-50 but not with AahII toxin, a representative of another antigenic group of scorpion toxins (El Ayeb et al., 1983b). This strongly suggests that BotXIV belongs to the same antigenic group as BotI.

No toxicity was observed when BotXIV was injected into mice, but significant toxicity towards insects was measured. This finding was confirmed by electrophysiological studies of cockroach axons. Recombinant toxin induced progressive prolongation of the evoked action potential under current-clamp conditions. Furthermore, under voltage-clamp conditions, recombinant toxin induced an increasing development of a maintained sodium current. This activity was similar to that observed for excitatory α-type toxins (Pelhate and Zlotkin, 1982; Eitan et al., 1990).

Comparison of the deduced amino acid sequence of BotXIV with those of other scorpion insect toxins revealed that BotXIV displays several important and unusual sequence differences, which could explain its moderate toxicity towards insects. Thus, when aligned for maximum similarity with insect-toxins, including BotIT1, BotIT4/5 (Borchani, L., unpublished data), LqhαIT (Eitan et al., 1990) and BjIT2 (Zlotkin et al., 1991), only 32–44% of the positions were identical. However, from these amino-acid-sequence data, no feature appears to correlate with the moderate insect toxicity of BotXIV. Data from directed mutagenesis of the closely related recombinant insect toxin LqhαIT (Zilberberg et al., 1995) showed that simultaneous substitutions of residues +8 to +10 (-Lys-Asn-Tyr-) by -Asp-Asp-Val-, led to a reduced toxic activity by several orders of magnitude. BotXIV has at these positions a -Gln-Pro-His- sequence, which differs from those of potent α-type insect toxins.

Recombinant BotXIV protein is highly immunogenic in mice and is capable of induction of BotG-50 neutralizing antibodies. The neutralizing capacity of anti-BotXIV sera, produced in mice, was in the same range as commercial horse immune sera produced against BotG-50, whose neutralizing capacity varied between 60–270 μg crude Bot venom/ml. This result demonstrates the importance of the present strategy to generate potent hybrid toxoids as previously demonstrated for snake toxins (Ducancel et al., 1989; Pillet et al., 1992). The serum raised against the hybrid that contained BotXIV was not only neutralizing but also confered *in vivo* protection for immunized mice against challenge with antigenically related mammal toxins. This suggests that the structure adopted by the toxin moiety within the hybrid protein ressembles that of the native toxin. Furthermore, it indicates that efficient immune protection can be achieved against structurally related potent mammal toxins by injection of low doses of a recombinant insect toxin. This strategy described here is particularly favorable for development of serotherapy against scorpion venoms since the immunologically active hybrid protein can be readily and efficiently produced in large amounts.

Our ultimate goal is to produce a recombinant construct that includes toxoids structurally and antigenically related to the potent toxins of *A. australis hector* and *B. occitanus tunetanus*, to progressively substitute the usual use of venoms as immunogen for serotherapy production and to confer immunoprevention against scorpion venoms.

We gratefully acknowledge Prof. K. Dellagi, Head of Institut Pasteur de Tunis and Prof. H. Rochat, Dr P. Bougis and Dr R. Kharrat, for encouragement and helpful advice. We would like to thank all members of the Département d'Ingénierie et d'Etudes des Protéines (DIEP) laboratories and, particularly, Françoise Bouet, for her help in amino-acid-sequence determination, Odile Trémeau, for HPLC purification of cleaved toxins, and Pascal Drevet, for fermentor operation. This research was supported by the *Institut National de la Santé et de la Recherche Médicale* grant no. 492 NS6, European Economic Community grant no. C11-CT93-001, and *accords d'échange* between the DIEP and Institut Pasteur de Tunis.

REFERENCES

Bougis, P., Rochat, H. & Smith, L. A. (1989) Precursors of *Androctonus australis* scorpion neurotoxins: structures of precursors, processing outcomes and expression of a functional recombinant toxin II, *J. Biol. Chem. 264*, 19259–19265.

Bouhaouala-Zahar, B., El Ayeb, M. & Karoui, H. (1996) *Construction et caractérisation d'une banque d'ADNc spécifique des toxines du scorpion* Buthus occitanus tunetanus, *Biologia (Tunis)*, in the press.

Boyer, H. W. & Roulland-Dussoix, D. (1969) A complementation analysis of the restriction and modification of DNA in *Escherichia coli*, *J. Mol. Biol. 41*, 459.

Boyot, P., Pillet, L., Ducancel, F., Boulain, J.-C., Trémeau, O. & Ménez, A. (1990) A recombinant snake neurotoxin generated by chemical cleavage of a hybrid protein recovers full biological properties, *FEBS Lett. 266*, 87–90.

Carbonell, L. F., Hodge, M. R., Tomalski, M. D. & Miller, L. K. (1988) Synthesis of a gene coding for an insect-specific scorpion neurotoxin and attemps to express it using baculovirus vectors, *Gene (Amst.) 73*, 409–418.

Catterall, W. A., Ray, R. & Morow, C. S. (1976) Membrane potential dependent binding of scorpion toxin to action potential sodium ionophore, *Proc. Natl Acad. Sci. USA 73*, 2683–2686.

Catterall, W. A. (1984) The molecular basis of neuronal excitability, *Science 223*, 653–661.

Catterall, W. A. (1986) Molecular properties of voltage-sensitive sodium channels, *Annu. Rev. Biochem. 55*, 953–985.

Chavez-Olortegui, C., Ait-Amara, D., Rochat, H., Diniz, C. & Granier, C. (1991) *In vivo* protection against scorpion toxins by liposomal immunization, *Vaccine 9*, 907–910.

Couraud, F., Jover, E., Dubois, J. M. & Rochat, H. (1982) Two types of scorpion toxins receptors sites, one related to the activation of the action potential sodium channel, *Toxicon 20*, 9–16.

Danse, J-M., Rowan, E. G., Gasparini, S., Ducancel, F., Vatanpour, H., Young, L. C., Poorheidari, G., Lajeunesse, E., Drevet, P., Ménez, R., Pinkasfeld, S., Boulain, J.-C., Harvey, A. L. & Ménez, A. (1994) On the site by which α-dendrotoxin binds to voltage dependent potassium channels: site-directed mutagenesis reveals that the lysine triplet 28–30 is not essential for binding, *FEBS Lett. 356*, 153–158.

Darbon, H., Weber, C. & Braun, W. (1991) Two dimensional ^{1}H nuclear magnetic resonance study of AaHIT, an anti-insect toxin from the scorpion *Androctonus australis hector*. Sequential resonance assignements and folding of the polypeptide chain, *Biochemistry 30*, 1836–1845.

Dee, A., Belagaje, R. M., Ward, K., Chio, E. & Lai, M. T. (1990) Expression and secretion of a functional scorpion insecticidal toxin in culture mouse cells, *Biotechnology (NY) 8*, 339–342.

De Lima, M. E., Martin, M. F., Diniz, C. R. & Rochat, H. (1986) *Tityus serrulatus* toxin VII bears pharmacological properties of both β toxin and insect toxin from scorpion venoms, *Biochem. Biophys. Res. Commun. 139*, 296–302.

Delori, P., Van Rietschoten, J. & Rochat, H. (1981) Scorpion venoms and neurotoxins: an immunological study, *Toxicon 19*, 393–407.

Ducancel, F., Boulain, J. C., Tremeau, O. & Menez, A. (1989) Direct expression in *E. coli* of a functionally active protein A-snake toxin fusion protein, *Protein Eng. 3*, 139–143.

Eitan, M., Fowler, E., Herrmann, R., Duval, A., Pelhate, M. & Zlotkin, E. (1990) A scorpion venom neurotoxin paralytic to insects that affects sodium current inactivation: purification, primary structure and mode of action, *Biochemistry 29*, 5941–5947.

El Ayeb, M., Delori, P. & Rochat, H. (1983a) Immunochemistry of alpha toxins: antigenic homologies checked with radioimmunoassays, *Toxicon 21*, 709–716.

El Ayeb, M., Martin, M., Delori, P., Bechis, G. & Rochat, H. (1983b) Immunochemistry of scorpion α-neurotoxins. Determination of the antigenic site number and isolation of highly enriched antibody specific to a single antigenic site of toxin II of *Androctonus australis Hector*, *Mol. Immun. 20*, 697–708.

El Ayeb, M. & Rochat, H. (1985) Polymorphism and quantitative variations of toxins in the venom of the scorpion *Androctonus australis Hector*, *Toxicon 23*, 755–760.

El Ayeb, M., Darbon, H., Bahraoui, E. M., Vargas, O. & Rochat, H. (1986) Differential effects of defined chemical modifications on antigenic and pharmacological activities of scorpion α and β toxins, *Eur. J. Biochem. 155*, 289–294.

Fontecilla-Camps, J. C., Almassy, R. J., Suddath, F. L., Watt, D. D. & Bugg, C. E. (1980) Three-dimensional structure of a protein from scorpion venom: A new structural class of neurotoxins, *Proc. Natl Acad. Sci. USA 77*, 6496–6500.

Fontecilla-Camps, J. C., Habersetze-Rochat, C. & Rochat, H. (1988) Orthorhombic crystals and three dimensional structure of the potent toxin II from the scorpion *Androctonus australis australis*, *Proc. Natl Acad. Sci. USA 85*, 7443–7447.

Gordon, D., Jover, E., Couraud, F. & Zlotkin, E. (1984) The binding of insect selective neurotoxin (AaHIT) from the scorpion venom to locust synaptosomal membranes, *Biochim. Biophys. Acta 778*, 349–358.

Gordon, D. & Zlotkin, E. (1993) Binding of a scorpion toxin to insect sodium channels is not dependent on membrane potentiel, *FEBS Letts 315*, 125–128.

Grégoire, J. & Rochat, H. (1983) Covalent structure of toxin I and II from the scorpion, *Toxicon 21*, 153–162.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids, *J. Mol. Biol. 166*, 557–580.

Hille, B. (1992) Ionic channels of excitable membranes, Sinauer Associates, Sunderland MA.

Hodgson, D., Gasparini, S., Drevet, P., Ducancel, F., Bouet, F., Boulain, J.-C., Harris, J. B. & Ménez, A. (1993) Production of recombinant notechis 11'2L, an enzymatically active mutant of a phospholipase A_2 from *Notechis scutatus scutatus* venom, as directly generated by cleavage of a fusion protein produced in *Escherichia coli*, *Eur. J. Biochem. 212*, 441–446.

Jover, E., Couraud, F. & Rochat, H. (1980) Two types of scorpion neurotoxin characterized by their binding to two separate receptor sites on

rat brain synaptosomes, *Biochem. Biophys. Res. Commun. 95*, 1607–1614.

Kopeyan, C., Mansuelle, P., Martin-Eauclaire, M. F., Brando, T., Bahraoui, E. M., Rochat, H. & Granier, C. (1990) Primary structure of anti-insect toxins isolated from the venom of *Leiurus quinquestriatus quinquestriatus*, *FEBS Lett. 261*, 423–426.

Kopeyan, C., Mansuelle, P., Martin-Eauclaire, M. F., Rochat, H. & Miranda, F. (1993) Characterization of toxin III of the scorpion *Leirus quinquestriatus quinquestriatus*: a new type of toxin highly toxic both to mammals and insects, *Nat. Toxins 1*, 308–312.

Kozak, M. (1984) Compilation and analysis of sequences upstream from the translation start site in eucaryotic mRNAs, *Nucleic Acids Res. 12*, 857–872.

Lester, D., Lazarovici, P., Pelhate, M. & Zlotkin, E. (1982) Two insect toxins from the venom of the scorpion *Buthus judaicus*: purification, characterization and mode of action, *Biochim. Biophys. Acta 701*, 370–381.

Löwenadler, B., Jansson, B., Paleus, S., Holmgren, E., Nilsson, B., Moks, T., Palm, G., Josephson, S., Philipson, L. & Ulhén, M. (1987) A gene fusion system for generating antibodies against short peptides, *Gene (Amst.) 58*, 87–97.

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor NY.

Martin-Eauclaire, M. F., Sogaard, M., Ramos, C., Cestèle, S., Bougis, P. E. & Svensson, B. (1994) Production of active, insect-specific scorpion neurotoxin in yeast, *Eur. J. Biochem. 223*, 637–645.

Miranda, F., Kupeyan, C., Rochat, H., Rochat, C. & Lissitzky, S. (1970) Purification of animal neurotoxin. Isolation and characterization of eleven neurotoxins from the venoms of the scorpions *Androctonus australis Hector*, *Buthus occitanus tunetanus* and *Leirus quinquestriatus quinquestriatus*, *Eur. J. Biochem. 16*, 514–523.

Nilsson, B., Moks, T., Jansson, B., Abrahmsèn, L., Elmblad, A., Holmgren, E., Henrichson, C., Jones, T. A. & Ulhén, M. (1987) A synthetic IgG-binding domain based on staphylococcal protein A, *Protein Eng. 1*, 107–113.

Okayama, H. & Berg, P. (1983) A cDNA cloning vector that permits expression of cDNA inserts in mammalian cell, *Mol. Cell. Biol. 3*, 280–289.

Pang, S. Z., Obbrhaus, S. M., Rasmussen, J. L., Knipple, D. C., Bloomquist, J. R., Dean, D. H., Bowman, K. D. & Sanford, J. C. (1992) Expression of a gene encoding a scorpion insectotoxin peptide in yeast, bacterial and plants, *Gene (Amst.) 116*, 165–172.

Pelhate, M. & Pichon, Y. (1974) Selective inhibition of potassium current in the giant axon of the cockroach, *J. Physiol. (Lond.) 242*, 90–91.

Pelhate, M. & Zlotkin, E. (1981) Voltage dependent slowing of the turn off of Na^+ current in the cockroach giant axon induced by the scorpion venom 'insect toxin', *J. Physiol. (Lond.) 319*, 30–31.

Pelhate, M. & Zlotkin, E. (1982) Actions of insect toxin and other toxins derived from the venom of scorpion *Androctonus australis* on isolated giant axons of the cockroach *Periplaneta americana*, *J. Exp. Biol. 97*, 66–77.

Pelhate, M. & Sattelle, D. B. (1982) Pharmacological properties of insects axons: a review, *J. Insect Physiol. 28*, 889–903.

Pichon, Y. & Boistel, J. (1967) Current-voltage relations in the isolated giant axons of the cockroach under voltage clamp conditions, *J. Exp. Biol. 47*, 343–355.

Pillet, L., Leonetti, M., Ducancel, F., Boulain, J.-C. & Ménez, A. (1992) Three distinct approaches to the generation of toxic protein antisera, *Rec. Adv. Toxinol. Res. 1*, 19–35.

Pillet, L., Trémeau, O., Ducancel, F., Drevet, P., Zinn-Justin, S., Pinkasfeld, S., Boulain, J-C. & Ménez, A. (1993) Genetic engineering of snake toxins: role of invariant residues in the structural and functional properties of a curaremimetic toxin, as probed by site-directed mutagenesis, *J. Biol. Chem. 268*, 909–916.

Proudfoot, N. J. & Brownlee, G. G. (1976) 3′ Non-coding region sequences in eucaryotic messenger RNA, *Nature 263*, 211–214.

Reed, L. & Muench, H. (1938) A simple method of estimating fifty percent end point, *Am. J. Hyg. 27*, 493–497.

Rochat, H., Bernard, P. & Couraud, F. (1979) Scorpion toxins: chemistry and mode of action, in *Adv. cytopharmacol. 3*, 325–330.

Rosenthal, J. A., Hsu, S. H., Schneider, D., Gentile, L. N., Messier, N. J., Vaslet, C. A. & Hawrot, E. (1994) Functional expression and site-directed mutagenesis of a synthetic gene for α-bungarotoxin, *J. Biol. Chem. 269*, 11178–11185.

Sanger, F., Nichlen, S. & Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors, *Proc. Natl Acad. Sci. USA 74*, 5463–5467.

Trémeau, O., Lemaire, C., Drevet, P., Pinkasfeld, S., Ducancel, F., Boulain, J.-C. & Ménez, A. (1995) Genetic engineering of snake toxins: the functional site of erabutoxinA, as delineated by site-directed mutagenesis, includes variant residues, *J. Biol. Chem. 270*, 9362–9369.

Vargas, O., Martin, M. F. & Rochat, H. (1987) Characterization of six toxins from the venom of the Moroccan scorpion *Buthus occitanus mardochei*, *Eur. J. Biochem. 162*, 589–599.

Walther, C., Zlotkin, E. & Rathmayer, W. (1976) Action of different toxins from *Androctonus australis hector* on a locust nerve muscle preparation, *J. Insect Physiol. 22*, 1187–1194.

Woods, D. (1984) Screening of cDNA library, *Focus (Idaho) 6*, 3–4.

World Health Organization (1981) Progress in the characterization of venoms and standardization of antivenoms, *Publication N°58*, Genève.

Zilberberg, N., Gordon, D., Zlotkin, E., Pelhate, M., Chejanovsky, N. & Gurevitz, M. (1995) Expression of an active alpha scorpion neurotoxin using *Escherichia coli*, insect cells and *Lepidopterous Larvae*: structure-activity relationship and future applications, *Toxicon 33*, 269.

Zlotkin, E., Miranda, F., Kopeyan, C. & Lissitzky, S. (1971 a) A new toxic protein in the venom of the scorpion *Androctonus australis hector*, *Toxicon 9*, 9–13.

Zlotkin, E., Rochat, H., Kopeyan, C., Miranda, F. & Lisstzky, S. (1971 b) Purification and properties of the insect toxin from the venom of the scorpion *Androctonus australis hector*, *Biochimie (Paris) 53*, 1073–1078.

Zlotkin, E., Kadouri, D., Gordon, D., Pelhate, M., Martin, M. F. & Rochat, H. (1985) An excitatory and depressant insect toxins from scorpion venom-both affect sodium conductance and possess a common binding site, *Arch. Biochem. Biophys. 240*, 870–887.

Zlotkin, E., Eitan, M., Bindokas, V. P., Adams, M. E., Moyer, M., Burkhart, W. & Fowler, E. (1991) Functional duality and structural uniqueness of depressant insect-selective neurotoxins, *Biochemistry 30*, 4814–4821.